DETECTION AND CHARACTERISATION OF METALLO BETA LACTAMASE PRODUCTION IN *PSEUDOMONAS AERUGINOSA* BY PHENOTYPIC AND MOLECULAR METHODS FROM CLINICAL ISOLATES IN A TERTIARY CARE HOSPITAL

DISSERTATION SUBMITTED TO

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE IN MICROBIOLOGY

(Branch IV) M. D. (MICROBIOLOGY)

of

THE TAMIL NADU DR. M. G. R MEDICAL UNIVERSITY

CHENNAI- 600032

DEPARTMENT OF MICROBIOLOGY

TIRUNELVELI MEDICAL COLLEGE

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APRIL 2015

CERTIFICATE

This is to certify that the Dissertation "**DETECTION AND CHARACTERISATION OF METALLO BETA LACTAMASE PRODUCTION IN *PSEUDOMONAS AERUGINOSA* BY PHENOTYPIC AND MOLECULAR METHODS FROM CLINICAL ISOLATES IN A TERTIARY CARE HOSPITAL**" presented herein by **Dr. V.G. SRI DEVI** is an original work done in the Department of Microbiology,Tirunelveli Medical College Hospital, Tirunelveli for the award of Degree of M.D.(Branch IV) Microbiology under my guidance and supervision during the academic period of 2012-2015.

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Tirunelveli-627011

CERTIFICATE

This is to certify that the dissertation entitled **"DETECTION AND CHARACTERISATION OF METALLO BETA LACTAMASE PRODUCTION IN *PSEUDOMONAS AERUGINOSA* BY PHENOTYPIC AND MOLECULAR METHODS FROM CLINICAL ISOLATES IN A TERTIARY CARE HOSPITAL"** submitted by **Dr. V.G. SRI DEVI** to the Tamilnadu Dr. M.G.R Medical University, Chennai, in partial fulfillment of the requirement for the award of M.D. Degree Branch – IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

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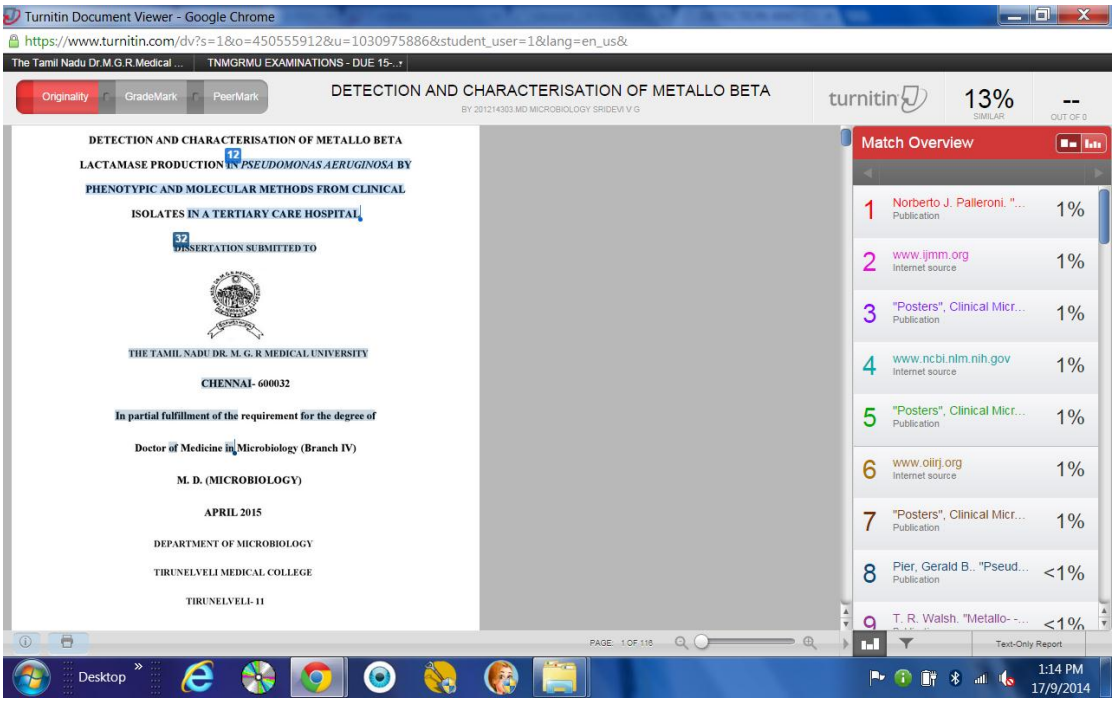
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I, **Dr. V.G. SRI DEVI** declare that, I carried out this work on **"DETECTION AND CHARACTERISATION OF METALLO BETA LACTAMASE PRODUCTION IN *PSEUDOMONAS AERUGINOSA* BY PHENOTYPIC AND MOLECULAR METHODS FROM CLINICAL ISOLATES IN A TERTIARY CARE HOSPITAL"** at the Department of Microbiology, Tirunelveli Medical College, I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in India or abroad.

This is submitted to the Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

Place: Tirunelveli **Dr. V.G. Sri Devi**

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CONTENTS

LIST OF ABBREVATIONS

P.aeruginosa	*Pseudomonas aeruginosa*
P.putida	*Pseudomonas putida*
MBL	Metallo betalactamase
Opr	Outer membrane porin
CLSI	Clinical Laboratory Standards Institute
CDT	Combined Disk Synergy Test
DDST	Double Disk Synergy Test
EDTA	Ethylene Diamine Tetra Acetic Acid
E test	Epsilometer test
MIC	Minimum Inhibitory Concentration
MEM	Meropenem
MP	Meropenem
MPI	Meropenem with EDTA
CFU	Colony Forming Unit
MHA	Muller Hinton Agar
ATCC	American Type Culture Collection
MRPA	Meropenem Resistant *Pseudomonas Aeruginosa*
MSPA	Meropenem Sensitive *Pseudomonas Aeruginosa*
NDM	NewDelhi metallobetalactamase

IMP	Imipenemases
VIM	Verona integrin encoded metallo β lactamase
SPM	Sao Paulo metallo β lactamase
GIM	German imipenemase
SIM	Seoul imipenemase
bla	Betalactamase
CSF	Cerebrospinal Fluid
μg	microgram
PCR	Polymerase chain reaction
DNA	Deoxy Ribonucleic Acid
IC	Internal Control
CT	Cross Threshold

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ABSTRACT

DETECTION AND CHARACTERISATION OF METALLO BETA LACTAMASE PRODUCTION IN *PSEUDOMONAS AERUGINOSA* BY PHENOTYPIC AND MOLECULAR METHODS FROM CLINICAL ISOLATES IN A TERTIARY CARE HOSPITAL.

INTRODUCTION:

P.aeruginosa is an opportunistic pathogen associated with a range of nosocomial infections. It flourishes as a saprophyte, with innate resistance to many antibiotics. In addition to its innate resistance, acquired resistance is particularly associated with indiscriminate use of antimicrobials. Carbapenams is the last resort drug used against this organism isolated from patients. Resistance to carbapenams has emerged by various mechanisms; one growing factor leading to the resistance is the production of metallo beta lactamases. With worldwide increase in the occurrence and dissemination of MBLs, early detection is crucial, the benefits of which include timely implementation of strict infection control practices and treatment with alternative antimicrobials.

OBJECTIVE:

- To detect the prevalence of MBL production in *Pseudomonas aeruginosa* isolates in Tirunelveli Medical College.
- To detect the MBL production in *Pseudomonas aeruginosa* isolates by phenotypic and genotypic methods.
- To evaluate various phenotypic methods in the detection of MBL.

METHODS AND MATERIALS:

This study will be conducted in the Department of Microbiology in Tirunelveli medical college hospital after approval from the institutional ethical committee. A total of 100 consecutive non repetitive clinical isolates of *P.aeruginosa* are subjected to three different phenotypic methods such as combined disc test (CDT), double disc synergy test (DDST), MBL E test using Meropenem and confirmed genotypically by RT-PCR for the presence of *bla* IMP and *bla* VIM gene.

RESULTS:

Out of 100 *P.aeruginosa* isolates, 21 were resistant to Meropenem. Out of 21 Meropenem resistant *P.aeruginosa* isolates 14 were detected as MBL producer by CDT, 12 and 10 were detected as MBL producer by DDST and MBL E test respectively. The PCR detected 10 isolates as MBL producer and all of them were positive for *bla* VIM gene and no *bla* IMP gene was detected in any of the isolates.

CONCLUSION:

This study shows that DDST is the simple and cost effective method in the detection of MBL and it should be confirmed with the MBL E test if PCR is not available in the clinical laboratory settings.

Keywords*: P.aeruginosa*, Meropenem, MBL, Phenotypic methods, PCR

1. INTRODUCTION

Pseudomonas aeruginosa are aerobic non-spore-forming, gram negative rods motile with the help of one or more polar flagella can grow normally using minimal nutritional components. Many of them are saprophytes and others are opportunistic pathogens of humans. *Pseudomonas aeruginosa* has been found in many things used in laboratories and medical practice.

P.aeruginosa can survive strict environmental conditions and shows intrinsic resistance towards many antimicrobials which facilitate the organism to survive in the hospital setting. Within the hospital, the colonization of *P. aeruginosa* is seen mostly in the moist surfaces of patients on the axilla, ear, and perineum. It is also isolated from other moist environments such as in water sinks and drains, toilets and showers. The equipment used in the hospital that comes in contact with water, such as mops, respiratory ventilators, cleaning solutions can also form the sources of *P. aeruginosa*[1].

Hence it is the most common opportunistic pathogen that requires compromised host defense to establish infection and is considered as the leading cause of hospital acquired infections. In normal individuals infection is usually associated with the disruption or bypass of the defense mechanism provided by the epidermis for instance burns, punctured

wounds, usage of contaminated needles and trauma to the eyes with contaminated contact lenses which results in infections of the skin and bone, septicemia, wound and eye infections[2].

For immunocompromised patients the infections due to *P.aeruginosa* are severe and life threatening. In cystic fibrosis patients these organisms have predilection to the respiratory tract and cause severe pneumonia. It may cause invasive malignant otitis externa in diabetic patients and in any condition which results in leukopenia, *P.aeruginosa* assumes an opportunistic pathogenic role[3]. There is also a problem of community acquired pseudomonas infection due to the exposure to the moist surfaces like swimming pools and other types of tubs[4].

As it is the leading cause of nosocomial infections and shows intrinsic and acquired resistance towards many antimicrobials, it increases the morbidity and mortality of the hospitalized patients[5]. Hospital acquired infections due to multidrug resistant *P. aeruginosa* have been related with increased hospital expenses and poorer clinical outcomes[6]. The resistance pattern in *P. aeruginosa* includes reduced permeability of the cell wall due to loss of OprD porin ,extracellular chromosomal and plasmid-mediated enzyme production like Metallo β-lactamases, aminoglycosidases and cephalosporinases, modification in antibiotic-binding protein sites, the up-regulation of an active efflux mechanism which drives out antibiotic from the cell[7]. Although the antibiotic resistance in *P.aeruginosa* is caused by

multiple mechanisms, one major factor leading to resistance is the production of metallo beta lactamases. Metallo beta Lactamases (MBL) hydrolyses all beta lactams including carbapenems except Aztreonam.

The prevalence of MBL producing *P. aeruginosa* ranges from 8 – 14% in various parts of India[8]. The resistance to the last resort drug Carbapenem due to the production of MBL is the emerging problem to the clinicians in treating the *P. aeruginosa* infections. Hence there should be urgency in detecting the resistance in *P. aeruginosa* in clinical laboratory.

There are different methods to detect phenotypically the production of the MBL production in the clinical laboratory. But there are no formulated guidelines from the CLSI for the detection of the metallo beta lactamase producing *P. aeruginosa*. The various phenotypic methods that are available to detect the MBL include, combined disc test (CDT), double disc synergy test (DDST), MBL E test, Modified hodge test. The genotypic method to detect and confirm MBL is the PCR (Polymerase Chain Reaction) which identifies the common genes like IMP and VIM encoding for MBL production. The molecular detection of the metallo beta lactamase using PCR is available only in the reference center is the limitation regarding the gene detection.

Early detection is critical and the benefits of which include timely execution of strict infection control practices, formulating an effective

antibiotic strategy to prevent the spread of these MBL producing strains, and treatment with alternative antimicrobials.

Hence the present study is intended to detect the presence of MBL producing *P. aeruginosa* and the susceptibility pattern of these strains to other antibiotics in clinical isolates in our hospital using both phenotypic and genotypic tests. All the *Pseudomonas aeruginosa* isolates are subjected to three different phenotypic methods such as combined disc test (CDT), double disc synergy test (DDST) and MBL E test using Meropenem disc and EDTA as chelator to find out their effectiveness in the detection of the production of MBL. The Meropenem disc is used to perform the phenotypic tests, as stated in the CLSI guidelines (2013) that the Imipenem disc performs poorly as a screen for carbapenemases. The PCR is also performed to check the presence of *bla*IMP and *bla*VIM gene responsible for the production of MBL.

2. AIMS AND OBJECTIVES

- To detect the prevalence of MBL production in *Pseudomonas aeruginosa* isolates in Tirunelveli Medical College.
- To detect the MBL production in *Pseudomonas aeruginosa* isolates by phenotypic and genotypic methods.
- To evaluate various phenotypic methods in the detection of MBL.
- To assess antibiotic susceptibility pattern of *Pseudomonas aeruginosa*.

3. REVIEW OF LITERATURE

3.1 HISTORY

Pseudomonas aeruginosa first documented as the source of 'blue pus' in wounds and hence named Bacillus pyocyaneus by Gessard. The name was altered by S edillot in to *Pseudomonas pyocyanea* in 1850. Luke noted the rod shaped bacteria in the blue green pus from the surgical dressings and later Schroeter named it *Pseudomonas aeruginosa* in 1872. Osler in 1925 suggested that this organism is said to invade the damaged tissues rather the healthy tissue[1]. *P. aeruginosa* produces the pigment pyocyanin that gives rise to a typical green colony on solid media. The name *aeruginosa* was derived from the green hue seen in colonies of clinical isolates.

In 1960s *P. aeruginosa* emerged as a major human pathogen due to its ability to cause infections in immunocompromised and burn patients and in those who were in modern medical equipment. Since then, *P. aeruginosa* has become one of the most severe causes of nosocomial infections, particularly in the lung, wound infections, blood and urinary tract[4].

3.2 TAXONOMY:

Palleroni divided the *Pseudomonas* into five ribosomal RNA groups based on rRNA-DNA studies. Based on phenotypic characteristics the pseudomonads are separated into 7 groups by Gilardi namely *fluorescent, stutzeri, alcaligenes, pseudomallei, acidovarans, facilis-delafieldii and*

diminuta. The name *Pseudomonas* was reserved for rRNA group I, which included *Pseudomonas aeruginosa*. The species of *Pseudomonas* can be subdivided into two groups, the fluorescent and the non-fluorescent species. The new edition of Bergey's Manual of systematic bacteriology includes about 65 species in *Pseudomonas*[1].

RNA Group I:

Flouresent Group

P. aeruginosa

P. fluorescence

P. putida

Stutzeri Group

P. stutzeri

P. mendocina

CDC group Vb-3

Alcaligenes Group

P. alcaligenes

P. pseudoalcaligenes

Pseudomonas species group-1

Fluorescent Group:

In this group all the species produce a water soluble pigment called pyoverdin that fluoresces blue-green under long wavelength ultraviolet light. Although all three members of this group produce Pyoverdin, only one

species, *Pseudomonas aeruginosa*, produces the distinctive blue pigment pyocyanin which is water soluble. *Pseudomonas aeruginosa* is the pseudomonad most frequently recovered from clinical specimens[9].

3.3 MORPHOLOGY:

Pseudomonas aeruginosa are aerobic gram negative rods. They are non-spore-forming, straight or slightly curved rods measuring 0.5 to 1.0 by 1.5 to 5.0 µm. They are motile, with single polar flagella.

3.4 CULTURAL CHARACTERISTICS:

Pseudomonas aeruginosa is nutritionally adaptable and grows readily on most common diagnostic media. They have a strictly aerobic respiratory metabolism and uses oxygen as the terminal electron acceptor. The nitrate can also be used as an alternative electron acceptor and grows anerobically. They are oxidase positive, catalase positive. The growth can occur in the temperature between 5 and 42^0 C and the optimum temperature is 37^0C. The optimum pH ranges from 7.4-7.6. The cultures produce a characteristics grape or earthy like smell of acetaminophenone.

On nutrient agar, six distinct colonial types of *Pseudomonas aeruginosa* are seen[10].

Type I: Large rough colonies surrounded by serrated skirts of growth.

Type II: Coliform like, Small, smooth domed colonies.

Type III: Small rough colonies

Type IV: Small rugose colonies

Type V: Mucoid Alginate producing colonies

Type VI: Dwarf colonies.

The type 2 colonies are most commonly isolated from the environmental sources and the type 5 is frequently isolated from respiratory secretions from cystic fibrosis patients.

On blood agar, a flat spreading colony with serrated edges often shows metallic sheen. It produces beta hemolytic colonies.

On Mac-conkey agar, colourless colonies which do not ferment lactose are produced.

It forms a dense turbidity with a surface pellicle in the broth.

3.5 PRODUCTION OF PIGMENTS:

The characteristic feature of the *P. aeruginosa* is the production of pigments. These pigments diffuse into the culture media as they are water-soluble.

Types of pigments produced by *P. aeruginosa:*

- Pyocyanin
- Pyorubin
- Pyomelanin
- Pyoverdin

Pyocyanin is the blue phenazine pigment which is characteristic of *Pseudomonas aeruginosa*. It is also enhanced by the addition of 10% of egg white to the complex media. Pyocyanin is water soluble, and the colour

depends on the pH. The presence of this pigment and a characteristic fruity odor are the valuable characters for the preliminary identification.

Pyorubrin is a red pigment and the addition of glutamate enhances its production. Pyomelanin is a brown pigment which is produced from aromatic amino acids such as tyrosine or phenylalanine. The pyoverdin is of three types and it enhances the virulence of the organism. This pigment is used for the purpose of typing (siderotyping).

Some of the strains produce pigments like phenazine-1-carboxylate, dihydroxyphenazine-1-carboxylic acid, chlororaphin and oxychlororaphin.

3.6 BIOCHEMICAL REACTIONS:

Oxidase positive, does not ferment carbohydrates, glucose is utilized oxidatively forming acid only. It appears inert. Indole and H_2S are not produced.

Voges – Proskauer and Methyl red reactions are negative. Nitrates are reduced to nitrites and to gaseous nitrogen.

Catalase, oxidase and Arginine dihydrolase tests are positive. Lactose, sucrose and mannitol are not fermented but xylose is fermented.

On Triple sugar iron agar alkaline slant and alkaline butt are produced with no gas and H_2S production.

Citrate is utilized and urea is not hydrolysed. Phenyl pyruvic acid is not produced and the gelatin is liquefied.

The selective medium for the isolation of *Pseudomonas aeruginosa* is the cetrimide agar in which the cetrimide acts as detergent which inhibits most bacteria and enhances the pigment production.

3.7 Minimum requirements for the definitive diagnosis of P.aeruginosa[9]:

- Gram negative rod
- Oxidase positive
- Typical smell i.e fruity grape like odour
- Recognizable colony morphology
    - Large green pigmented colonies with metallic sheen and beta hemolysis on blood agar.
    - On Mac conkey agar, colourless, non-lactose fermenting, colonies

3.8 TYPING METHODS:

The typing methods are used mainly for epidemiological purposes. There are three methods of typing namely, bacteriocin typing, serotyping and DNA restriction analysis.

3.8.1 Bacteriocin typing:

Bacteriocins are the proteins produced by a strain which is lethal to other strains of the similar bacteria. Pyocins are the bacteriocins produced by *P.aeruginosa.* There are three types of pyocins namely R, F, and S are

produced by *P.aeruginosa.* About 90% of the *P.aeruginosa* strains produce pyocins[10].

3.8.2 Serotyping:

The serotyping is done based on the reaction of heat stable O-antigen with a set of antisera. The antisera are prepared against the *Pseudomonas* cell suspensions.

3.8.3 DNA restriction analysis:

The endonucleases are used in the restriction analysis. *Pseudomonas* is rich in G+C pairs, and after digestion they will give more fragments. The separation is usually done by electrophoresis.

3.9 EPIDEMIOLOGY:

The primary habitat of *P. aeruginosa* is the environment and it is found in water, soil, and various types of vegetation. *P. aeruginosa* was isolated from the throat and stool in 7% and 24% respectively of healthy humans[4]. *P. aeruginosa* can grow with minimal nutritional requirements such as in distilled water and can tolerate temperatures as high as 45^0C to 50^0C. It grows even in antiseptic solutions with the help of divalent cations such as carbon. The minimal nutritional requirement for its growth contributes to its ecological success and its major role as opportunistic pathogen. Moisture is the serious factor which is associated with the human colonization. It colonizes in moist areas like axilla, perineum and ear. External sources of infection can be various materials, such as plants and

flowers, brought by visitors or health personnel that colonize the hospital environment like respiratory instruments, ventilators and in many equipment used in clinical laboratories and medical practice[4].

P. aeruginosa skin infections (Jacuzzi) are related to use of hot tubs and swimming pools are the common clinical presentations of community-acquired infection. The community- acquired *P. aeruginosa* infection is considered a minor problem when compared to the nosocomial infection.

Gastrointestinal (GI) colonization occurs secondary to the use of antibiotics that disrupt the normal microbial flora of the GI tract which sometimes can lead to aspiration results in the respiratory tract colonization.

The extended-wear of contact lenses has increased risk of *P. aeruginosa* ulcerative keratitis due to the presence of the organism in the lens solution.

Patients with burn wounds are at high risk for *P. aeruginosa* infection due to the loss of physical barrier of the intact dermis. Patients, who receive mechanical ventilation, become colonized with *P. aeruginosa* results in pneumonia known as ventilator associated pneumonia.

O.Oncul, *et al* from Iraq in his studies reported that the rate of infection with the *P.aeruginosa* is increased in burn patients[11].

The National Nosocomial Infections Surveillance system 1986-2003 recorded *P. aeruginosa* as the 2nd common cause of pneumonia, third most common cause of urinary tract infections. *P. aeruginosa* is a common cause

of nosocomial urinary tract infection that represents 7 % of all nosocomial infections. Community acquired urinary tract infections are not commonly caused by *P. aeruginosa*[12].

In the United States, the health care associated pneumonias accounted for about 18.1%, 9.5% of surgical site infections and 3.4% of septicemia in intensive care units are due to *P. aeruginosa* in the year 2003[4].

Invasive *P. aeruginosa* infection usually precedes a period of colonization in 50% of infected patients with an underlying risk factor for infection.

In hospitals the most common opportunistic pathogen is *P. aeruginosa.* This infection is a leading cause of morbidity and mortality of the patients. This increases the problem in the treatment of this infection.

Elizabeth B Hirsch *et al* reported that in a study with 136 patients, the mortality rate increases with the inappropriate and delay in the treatment of MDR *P. aeruginosa*[6].

3.10 PATHOGENESIS:

P. aeruginosa commonly requires a break in defenses to initiate infection. In burns and wound infections, *P. aeruginosa* grows better in dead or poorly perfused tissue, to allow it to seed the blood and devastate the immunity of the host[4].

The normal healthy eye is resistant to *P. aeruginosa* infection, but when the physical integrity of the corneal epithelium is broken, *P.*

aeruginosa turn out to be a major pathogen. The fundamental mechanism related to increased host susceptibility to *P. aeruginosa* infection is loss of tissue integrity.

Adherence of the organism to the host tissue is the first step in infection and it is mediated by adhesins like pili, flagella, and the extracellular polysaccharide slime which bind to the receptors. The receptors include sialic acid and N acetyl glucosamine and the attachment is enhanced by loss of surface fibronectin, which explains in part the susceptibility for debilitated persons. Entry of *P. aeruginosa* in the host occurs by the oral or respiratory route.

Host tissues provide a stressed environment for the organism with reduced availability of nutrients, oxygen, and other growth factors. The response of the microbe to the in vivo environment and the antimicrobial response of the host regulate the progression from colonization to infection and the disease. The virulence factors such as exotoxin A, exotoxin S, and elastase are directly injected into host cells by a special contact secretion system[3].

The dissemination from burn wounds is associated with Exoenzyme S which destructs the cytoskeleton of the cell. The elastase in the *Pseudomonas* attacks the elastin in the lung and blood vessels. The hemorrhagic destruction of the walls of blood vessels is the histologic feature of *Pseudomonas* infection.

The ability to form biofilms and the mechanism of quorum sensing plays an important role in the pathogenesis of the disease. The *P. aeruginosa* can grow anaerobically if nitrate is present and it is the significant pathogen of implanted medical devices such as intravenous catheters, orthopedic implants on which the organism can colonize and form a biofilm which disseminate systemically. The major components of a biofilm are a matrix composed of polysaccharide called a glycocalyx.

3.11 VIRULENCE FACTORS[9]:

P. aeruginosa produces numerous virulence factors in the form of enzymes and toxins that is responsible for its complex pathogenesis.

Exotoxin A (ETA) is the most toxic extracellular enzymes produced by *P. aeruginosa*. It inhibits the protein synthesis which is similar to that of the diphtheria toxin.

Alkaline proteases and elastase cause vascular lesions, necrosis and shedding of respiratory epithelial cells. These two metallo protease enzymes are inhibited by chelating agents.

Alginate is a capsular polysaccharide which is helpful in the adherence of the bacteria to the lung epithelium and forms biofilm. It resists phagocytosis and opsonisation killing by the host cells.

Pili which is responsible for the twitching motility, helps in adherence of the organism to GM-1 ganglioside. It also takes part in the biofilm formation. The siderophore receptors are present in the outer membrane

which enhances the microbial growth by providing iron as a source. It also has efflux pumps to remove antibiotics. The pyoverdine and pyochelin are the two siderophores produced by the *Pseudomonas aeruginosa*. The heat-labile phospholipase C and heat-stable rhamnolipid are the two hemolysins produced by *P. aeruginosa*.

There are three types of proteases secreted by the organism namely LasA protease, LasB elastase and alkaline protease which degrades host immune effectors like antibody and complement. Ferripyochelin and Pyocyanin are the secreted oxidative factors which produce reactive oxygen radicals that disrupt epithelial cell function and cause oxidative damage to tissues. It also damages the respiratory ciliary activity.

Lipopolysaccharide is endotoxic and antiphagocytic which causes sepsis syndrome, DIC and metabolic abnormalities.

Neuraminidase is the virulent enzyme that facilitates the binding of pili by removing the sialic acid residues from GM-1 ganglioside receptors. The Phospholipase C destructs the cytoplasmic membrane and pulmonary surfactant and results in the respiratory infection. The other factors like Leucocidin, inhibits the function of lymphocyte and neutrophils. The enterotoxin causes diarrhea.

The virulence factors depend upon the site and nature of infection.

- Proteases have a major role in corneal ulceration.

- Exotoxin and elastases are responsible for burns infections and septicemia.
- The Quorum sensing molecules and Alginate are related to the biofilm formation in chronic pulmonary colonization.
- Pyochelin and pyoverdin are the bacterial siderophores that enhances the virulence.

In Cystic Fibrosis, the mucoid colonies of *P. aeruginosa* strains are due to the increased production of a polysaccharide polymer known as Alginate. The pulmonary infection can also be caused by the virulence of flagella and fimbriae. The non pilus adhesins of *P. aeruginosa* adhere to the mucous surfaces of the lungs and increase the pathogenicity. The Pyocyanin pigment induces the release of interleukin-8 in cystic fibrosis that increases the intracellular oxidant stress.

3.11.1 Quorum Sensing[1]:

The communication system between the cells population in which the regulation of transcription of genes occurs only when a critical mass of the cell is achieved. The system of quorum sensing (QS) allows the organism to communicate with each other after the critical mass is reached.

The low-molecular-weight mediators of the quorum sensing response are synthesized and secreted. These molecular mediators are called auto inducers (AIs). The auto inducers diffuse through the cells of the bacterial community that induce gene transcription and virulence factor production.

There are three major interrelated quorum sensing systems are known to exist in *P. aeruginosa.* These include the las, rhl, and *Pseudomonas* quinolone system (PQS).

The expression of several virulence factors, including elastase and rhamnolid are regulated by the quorum-sensing system of P. aeruginosa.

3.11.2 Biofilm as a mechanism of resistance[4]:

The other mechanism that involves in the virulence is the biofilm formation that protects the organism from many antibiotics. Biofilm plays a major role in the pathogenesis of chronic infections such as osteomyelitis and cystic fibrosis.

The mechanism of resistance behind the biofilm formation include quorum sensing , decreased diffusion of antibiotics through the matrix polysaccharide alginate , synthesis of glucans that specifically bind antibiotics , and anaerobic growth of biofilm bacteria.

3.12 CLINICAL MANIFESTATIONS:

P. aeruginosa can produce any of the opportunistic infections. It causes infections in burns and wound, urinary tract, skin, eye, ear and respiratory infections which may progress to septicemia.

It may cause post-operative wound infections very commonly. A study by Prabhat ranjan et al on surgical wound infections reported that the most common (29.6%) bacteria isolated was *P. aeruginosa*[13]. Anupurba et

al in a similar study concluded that *P. aeruginosa* was isolated in 32% out of 940 clinical isolates from wound infections[14].

It is the common pathogen isolated in burn patients. Kalantar E, et al in his studies reported that swab from 176 burn patients, 100 swabs were turned to be *P. aeruginosa*[15].

Rajput A, et al, 2007 in his study with 154 isolates from burn patients, the most common organisms isolated were *Pseudomonas aeruginosa* (55%)[16].

In 1959-1963 the burn wound sepsis is the major cause of death in 60 % of the burns patients in US army[5]. In a study conducted in Iran by Leila Azmi et al said that the burn patients are more vulnerable to the colonization of *P. aeruginosa* in the environment. Among 717 samples taken from the burns, *P. aeruginosa* was isolated from 324 specimens. The mortality rate was 12% and all the patients who died had one culture positive with *P. aeruginosa* [17].

Pseudomonas aeruginosa is very invasive that frequently causes severe damage to the tissues in diabetic foot ulcers. Tamil Selvi Sivanmaliappan et al in their study showed that about 14.3 % of the pus collected from the diabetic foot ulcers are *P. aeruginosa*[18]. *P. aeruginosa* is also a common cause of otitis externa, including “swimmer’s ear” and “malignant” otitis externa in diabetic patients.

Pseudomonas aeruginosa is responsible for nosocomial pneumonia especially among patients who have been hospitalized in intensive care units and under mechanical ventilation. Ventilator associated pneumonia (VAP) caused by *Pseudomonas aeruginosa* remains a severe and dreaded complication [19].

In a study by Mayank Dwivedi et al showed that VAP was the most common infection among the hospital acquired infections due to *Pseudomonas aeruginosa* and it was about 63 (31.5%) patients out of 200 patients admitted in the ICU[20].

Crouch Brewer et al revealed that about 38% of the deaths are directly associated with the *P. aeruginosa* in the ventilated patients. Septic shock and multiple organ dysfunction syndromes are the main causes of death among these VAP patients[21].

It also causes osteomyelitis in environmentally contaminated wounds of compound fractures or nail puncture wounds of the foot and also in prosthesis. The etiologic agent in 4 to 6% of infected orthopedic devices was *Pseudomonas aeruginosa*[22].

P. aeruginosa causes pneumonia particularly in patients with neutropenia. Pulmonary infection in cystic fibrosis is a chronic infection with alveolar necrosis, vascular invasion, infarcts and bacteremia.

The organism can cause conjunctivitis, keratitis, or endophthalmitis when introduced into the eye by trauma. The people who wear contact

lenses for an extended period are at increased risk of *P. aeruginosa* ulcerative keratitis. Cheng KH et al in his study reviewed about 96 cases of microbial keratitis that wear contact lenses for a long period and stated that *P. aeruginosa* was the commonest organism isolated[23].

In *P. aeruginosa* bacteremia, cutaneous papules develop into necrotic ulcers called ecthyma gangrenosum due to the destruction of blood vessel walls by the organism. CNS infections are rare and are always secondary to head injury or surgical procedure[4].

3.13. TREATMENT OF INFECTIONS DUE TO *PSEUDOMONAS AERUGINOSA:*

P. aeruginosa is usually resistant to penicillin, ampicillin, tetracycline, chloramphenicol, sulfonamides, cephalothin, and the earlier aminoglycosides like streptomycin, kanamycin. The antibiotics with antipseudomonal activity include the newer aminoglycosides like gentamicin, tobramycin, and amikacin, ticarcillin, ureidopenicillins, third generation cephalosporins such as ceftazidime, cefepime and cefoperazone, quinolones, aztreonam and the carbapenems.

Carbapenems have a wider spectrum of activity against multiresistant *P. aeruginosa* isolates and are used as reserve drugs for the treatment of infections.

3.14. MECHANISM OF ANTIBIOTIC RESISTANCE:

This microorganism very often possesses various mechanisms to express the resistance towards almost all the available antibiotics.

3.14.1. Penicillins and Cephalosporins[24]:

The carbenicillin is semi-synthetic penicillin and was introduced in 1967 against *P. aeruginosa.* It has a carboxyl group in their side chains in order to alter the susceptibility to inactivating enzymes like β-lactamases and to enhance the effectiveness of antibacterial activity and the pharmacological properties of the drug.

The first cephalosporins obtained from *Cephalosporium acremonium* and the active nucleus is 7-aminocephalosporanic acid, which consists of a β-lactam ring fused to a dihydrothiazine ring. The side chains are added to produce semisynthetic compounds with wider range of antibacterial activity.

Cephalosporins are classified by generations based on general features of their antibacterial activity. The first and second generation cephalosporins are not active against the *Pseudomonas aeruginosa*. The ceftazidime and cefoperazone which belongs to the third generation cephalosporins have potent action against *Pseudomonas aeruginosa*.

3.14.1.1. Mechanism of Action:

Penicillins and cephlosporins inhibit bacterial growth by interfering with the transpeptidation reaction of bacterial peptidoglycan synthesis

which is a heteropolymeric component of the cell wall that gives stability to the organism.

3.14.1.2. Mechanism of Resistance[7]:

P. aeruginosa possesses an inducible chromosomal AmpC β-lactamase which causes resistant to penicillins, first generation and second-generation cephalosporins. It also confers resistance to cefotaxime and ceftriaxone.

The other mechanism of resistance to the β-lactam antibiotics is the presence of multiple efflux pumps in *P. aeruginosa* that can result in expulsion of β -lactams. The acquired resistance is by the β-lactamases that are encoded in plasmids, on transposons or integrons which confer resistance to antipseudomonal penicillins, ceftazidime and cefipime.

3.14.2 β-LACTAMASE INHIBITORS:

The molecules that inactivate the enzyme β-lactamase, and thereby prevent the destruction of β lactam antibiotics are called β-lactamase inhibitors. They are mostly active against plasmid-encoded β-lactamases that hydrolyze ceftazidime and cefotaxime. Clavulanic acid, sulbactam, tazobactam are the common β-lactamase inhibitors used against *P. aeruginosa* infections now a day.

3.14.3 MONOBACTAMS[7]:

Monobactam binds to penicillin binding protein 3 (PBP 3) of gram-negative aerobic organism like *P. aeruginosa*, thus inhibiting the bacterial

cell wall synthesis. Like carbapenems it is also stable against commonly occurring plasmid and chromosomally mediated β-lactamases. Aztreonam is the only monobactam available for clinical use.

3.14.4 Resistance Mechanism of Aminoglycosides, Quinolones and other antibiotics[7]:

There are various mechanisms responsible for the intrinsic resistance of the organism towards many antibiotics. The impermeability mutation due to the loss of the OprD porin that results in the resistance of aminoglycosides, quinolones and colistin. The aminoglycoside-modifying enzymes often encoded on transposons and integrons result in various combinations of resistance to gentamicin, tobramycin or amikacin.

These enzymes also carry resistance determinants for other classes of antibiotics such as sulfonamides, β-lactams and chloramphenicol. Along with the impermeability mutations these Aminoglycoside-modifying enzymes result in the broad-spectrum aminoglycoside resistance. It has been described that the broad-spectrum aminoglycoside resistance is due to a gene rmtA.

The intrinsic mechanism which includes efflux pump systems mediated by some regulatory genes can cause expulsion of β -lactams, fluoroquinolones, macrolides, sulfonamides, chloramphenicol, tetracycline, and trimethoprim.

The overproduction of the efflux pump is due to the upregulation of the mexR gene which can cause resistance to antibiotics such as quinolones, penicillins, cephalosporins and aztreonam.

3.14.5 CARBAPENEMS:

Carbapenems are distinctive of all classes of β-lactam antibiotics. They have a hydroxyl ethyl side chain at position 6 and lack sulphur or oxygen atom in the nucleus.

3.14.5.1 MECHANISM OF ACTION OF CARBAPENAMS:

Carbapenems bind to penicillin binding protein (PBP 1 and PBP 2) of gram-negative and gram-positive bacteria. They cause elongation of cell and lysis. They are not hydrolyzed by most plasmid or chromosomally mediated β-lactamases. The peculiar stereochemistry of the hydroxyethyl side chain gives stability against β-lactamases. The currently available carbapenems for clinical use are doripenem, ertapenem, imipenem, and meropenem.

3.14.5.2 MECHANISM OF RESISTANCE:

The resistance towards carbapenem is classified into enzymatic and non-enzymatic. The non-enzymatic reasons for the resistance towards carbapenem are,

3.14.5.2.1 Over- expression of efflux pumps:

The most commonly observed pump system in *Pseudomonas aeruginosa* is MexABOprM (Multidrug efflux system AB- Outer membrane protein M). It consists of the MexA, the MexB and the OprM exit portal.

The overproduction of the efflux pump is due to the upregulation or mutation in the mexR gene which results in the resistance to Meropenem. When the over-expression efflux pump coexists with the reduced outer membrane permeability then there develops resistance to other carbapenems also [25].

3.14.5.2.2 Diminished outer membrane permeability:

Carbapenems enter the *P.aeruginosa* through the outer membrane porin OprD. Due to the mutation in the OprD gene there will be loss of porins which results in the Imipenem resistance. The down regulation in the OprD outer membrane porin leads to reduced susceptibility to Meropenem but the β lactams are not affected. The loss of OprD porin or diminished expression is the frequent causes of the failure in the treatment with Imipenem[7].

In a study done by J.K. Lee et al in Korea concludes that mutation in OprD gene from P. aeruginosa isolates was linked with the down-regulation of OprD transcription and Imipenem resistance[26].

Carbapenem resistant but beta-lactam susceptible strains are mainly due to the diminished expression of OprD. But these are not seen in the multidrug resistant strains.

3.14.5.2.3 Enzymatic – Beta-lactamases:

β-lactamases are the enzymes with serine proteases at their active-site. β-lactamases destroy the amide bond of a β-lactam in a two-step

reaction. The positively charged residue of the enzyme attracts the negatively charged carboxylate group of the β-lactam antibiotic and binds with the help of hydrogen bonding. The β-lactam is acylated. A strategically positioned water molecule deacylates the β-lactam antibiotic and regenerates the enzyme β- lactamase[7].

3.14.5.2.3.1 Classification of β-lactamases:

β-lactamases are classified according to two properties, the functional and molecular level.

3.14.5.2.3.1.1 Functional classification:

Functional classification proposed by Bush-Jacoby in 1988 is widely accepted and it classifies the β-lactamases into four major functional groups.

- Group 1 cephalosporinases,
- Group 2 penicillinases,
- Group 3 metallo-β-lactamase,
- Group 4 is unclassified.

In the functional classification, carbapenemases are found principally in groups 2f and 3.

3.14.5.2.3.1.2 Molecular classification:

The structural classification by Ambler includes four molecular classes:

A) The Extended Spectrum Beta-Lactamases (ESBL) that are inhibited by clavulanic acid,

B) The Metallo-Beta-Lactamases.

C) The Cephalosporinases.

D) The Oxacillinases.

The enzymes in Classes A, C and D have serine in their active site. The MBLs bear zinc in their active site. The amide bond of the beta-lactam ring is cleaved by the serine enzymes to inactivate the antibiotic, while MBLs use one or two divalent Zn^{2+} cations to catalyze the same chemical reaction.

The carbapenemases in *P.aeruginosa* belong to Ambler class B, commonly referred to as metalloenzymes.

3.14.5.2.3.1.3 Bush Jacoby Classification:

They classified the beta lactamases into 4 groups (1-4) and 5 subgroups (a-f)

Group 1: involves cephalosporinases that resist to clavulanic acid, comparable with the class C in Ambler scheme.

Group 2: consists of penicillinases and cephalosporinases that are inhibited by clavulanic acid, comparable with the class A and D in Ambler classification. The TEM and SHV were originally classified in this group. Due to the increase in the number of TEM and SHV derived beta lactamases, this group as divided into 2 subclasses, 2a and 2b.

Subgroup 2a: composed of penicillinases.

Subgroup 2b: consists of broad spectrum beta lactamases which can inactivate penicillins and cephalosporins at the same rate.

There are sub-subgroups, 2be and 2br classified under the subgroup 2b.

Sub-subgroup 2be: is consisted of Extended spectrum beta lactamases that can hydrolyse the third generation cephalosporins like cefotaxime, ceftazidime and cefpodoxime. It also hydrolyzes the monobactams (aztreonam).

Sub-subgroup 2br: this group contains the enzymes that are resistant to clavulanic acid and sulbactam. This is named ‘r’ due to its reduced ability to bind to clavulanic acid and sulbactam. This group is called inhibitor-resistant TEM derivative enzymes but is still sensitive to tazobactam.

Subgroup c: this group was later separated from subgroup 2b which have the property to inactivate carbenicillin more efficiently than benzyl penicillin.

Subgroup 2d: this group of enzymes is able to hydrolyze cloxacillin and carbenicillin more than the benzyl penicillin and also inhibited by clavulanic acid.

Subgroup 2f: this group consists of serine based carbapenemases but is different from zinc based carbapenemases in group 3.

Group 3: this group consists of zinc based carbapenemases or metallobeta lactamases comparable with the Ambler class B. This is the only group

which requires the metal ion zinc for their catalytic activity. These enzymes hydrolyze penicillins, cephalosporins and carbapenems.

Group 4: is the group composed of penicillinases that resist to clavulanic acid and not comparable with any of the group in the Ambler class.

3.15 METALLO-β-LACTAMASE: (MBL)

The first metallo-β-lactamase isolated were chromosomal enzymes that were present in environmental and opportunistic bacteria. These chromosomal enzymes express serine β lactamase, inducible only after the exposure to β lactam antibiotics. The genes responsible for the chromosomal enzymes are not easily transferred.

There are some acquired or transferable families of these metallo enzymes which spread through plasmids or transposons between bacteria. The most common metallo β lactamase families include the VIM, IMP genes located in integrons. Transferable carbapenem resistance in a *P. aeruginosa* isolate was first discovered in Japan in 1990[27].

The important features of metallo β lactamase are listed below,

- Class B metallo β lactamases use a Zn^{2+} cation for hydrolysis of the β-lactam ring.
- They hydrolyse all β-lactam antibiotics like penicillins, cephalosporins and even carbapenems except monobactam.
- They are susceptible to ion chelators such as ethylene diamene tetraacetic acid (EDTA) and thiol compounds.

- They are not inhibited by the β-lactamase inhibitor such as clavulanic acid.

3.15.1 CHEMICAL NATURE OF MBLs[7]:

MBLs carry a set of amino acids with two zinc ions at their active site. The zinc ions have two water molecules which is essential for hydrolysis. The major zinc-binding site is histidine-X- histidine-X-aspartic acid (HXHXD). These two zinc binding sites function separately with the primary zinc binding site assisted by the secondary site. This is common to all types of MBLs except the class B2 enzymes. The class B2 enzymes have only one zinc ion at their active site.

These MBL enzymes contain a αββα site with a central β and two helices on each side. The zinc ions are held in place by three histidines molecule and a water molecule. The water molecule plays a critical role in catalysis of the antibiotics.

The zinc in the active site positions the β-lactam bond to enable nucleophilic attack by zinc-bound water and hydroxides. These enzymes are resistant to the inhibitors such as clavulanic acid and sulbactum they will not hydrolyze aztreonam particularly well.

3.15.2 CLASSIFICATION OF METALLO-β-LACTAMASES:

3.15.2.1 Depending on the zinc requirement:

Metallo β lactamases can be clustered into three different subclasses (B1, B2, and B3) depending on their requirements for zinc.

B1 enzymes: They are fully active with one or two zinc ions. These include IMP-1, VIM-2, and CcrA.

B2 enzymes: These enzymes are inhibited by the addition of second zinc ion. The example is CphA.

B3 enzymes: These enzymes require two zinc ions. Eg: L1

3.15.2.2 Depending on the carbapenem hydrolyzing capacity:

On the basis of carbapenem hydrolyzing capacity the class B enzymes are divided into three groups

3a: It has a broad spectrum activity. The examples include BCII, IMP-I, Ccr A, VIM, GIM, SPM-1. It has property of binding of two zinc atoms for optimal hydrolysis. Three molecules of histidine are present in the one binding site of the zinc and asparagine, cysteine and histidine are present in the second binding site of zinc in the enzyme.

3b: It has preferential activity towards carbapenem. The examples include Cph A, Sfh-1. It has two molecules of histidine and one molecule of asparagine in the first binding site of the zinc atom. The addition of second zinc atom is inhibitory to the catalytic activity of the enzyme.

3c: It has high specificity for hydrolyzing cephalosporins but has poor activity against carbapenem. It has three histidine molecules in the first binding site and two histidine and a asparagine molecule in the second binding site which will be helpful in the optimum hydrolysis of the antibiotics.

3.15.2.3 Depending on the location of the MBL gene:

According to the location of MBL gene they are classified into two types,

- Transferable MBLs
- Chromosomally mediated MBLs

3.15.2.3.1 Chromosomally Mediated MBLs:

Most of the metallo β lactamases are chromosomally encoded. Their expression can be constitutive or inducible. In *B. cereus, Stenotrophomonas maltophilia*, *Aeromonas hydrophila,* and *Aeromonas jandaei* the metallo β lactamases are inducible.

Fortunately, these bacteria are generally opportunistic pathogens and are not frequently associated with serious nosocomial infections. But these bacteria show high level resistant to β lactams. As these MBL genes are chromosomally encoded, they are not easily transferred.

3.15.2.3.2 TRANSFERABLE MBLs[28]:

The Transferable MBLs are classified into five types according to the gene constituent,

i. IMP (“Imipenemases”)
ii. VIM (“Verona integron-encoded metallo β lactamase”)
iii. SPM (“Sao Paulo metallo β lactamase”)
iv. GIM (“German imipenemase”)
v. SIM (“Seoul imipenemase”).

The Transferable MBLs are located in the integrons. Integrons are the genetic elements of variable length that consists of three regions namely, 5'conserved region, 3' conserved region, and a variable region and has 59 conserved integrase gene *(int),* an integration site for the gene cassette, *attI* (attachment) and mobile antibiotic resistance genes called cassettes respectively.

By using a site-specific recombination system, integrons capture antibiotic resistance gene cassettes. There are about five distinct integron classes are associated with antibiotic resistance gene cassettes. Three major classes of integrons class1, class2 and class 3 have been described for gram-negative bacteria.

Integrons which have β lactamases are commonly present in *A. baumannii, P. aeruginosa,* and other species of gram negative bacteria included in Ambler class A, B, and D β lactamases.

The integrons which carry multiple resistance gene cassettes can be readily mobilized. Hence they are significant in the spread of antibiotic resistance.

Integrons are found to be an important source for the spread of *bla* gene. The *bla* gene types like IMP-1 to IMP-4, IMP-6 to IMP-8, IMP-12, VIM-1, VIM-2, and GIM-1 are also included in integron encoded β lactamases.

3.15.2.3.2.1 IMP type MBLs:

IMP (Imipenemase) was the first transferable MBL isolated from *P.aeruginosa* strain GN17203 in Japan during 1988. It has a high MIC for imipenem and also exhibited resistance to cephalosporins such as ceftazidime. These resistance alleles were set up on a mobile conjugative plasmid which can be easily transferred to other *P.aeruginosa* strains. These alleles were found in the class 1 integron.

The same identical gene isolated in *Serratia marcescens* Tn9106 from an UTI patient, three years later was found in class 3 integron.

To date there are up to 18 members in the IMP family listed on the β lactamases nomenclature.

Johann D. D. Pitout et al 2004, in his studies obtained about 2% of the IMP gene from 241 clinical strains of imipenem resistant *P. aeruginosa* from the centralized laboratory in the Calgary Health Region[29].

In a study Fallah F, et al out of 83 imipenem resistant strains 48 were MBL producers. PCR and Sequencing methods proved that these 48 isolates were positive for *bla*IMP-1 genes, none of them were positive for *bla*VIM genes[30].

Sepehriseresht S et al in his study reported that from the 483 isolates of carbapenam resistant and intermediate *P.aeruginosa* strains in burn patients from Iraq, ninety-four isolates had VIM and IPM genes in their PCR results.[31]

A study by Dong et al. reported that among 146 strains of multi drug resistant *Pseudomonas aeruginosa*, 46 (31.5%) were positive for the MBL production and carried the *bla*IMP and *bla*VIM gene.[32]

Nam Hee Ryoo et al in his study stated that 31 *Pseudomonas aeruginosa* isolates were producing the MBL enzyme and 21 among them were positive for IMP gene and 10 were positive for VIM gene[33].

3.15.2.3.2.2 VIM type MBLs: ("Verona integron-encoded metallo β lactamase")

The VIM type of enzymes is the second main group of acquired MBLs. VIM-1 was first described in Verona in 1997, from a *P. aeruginosa* isolate. It was found to be resistant to most of the β lactams and also the aztreonem. The resistance to the aztreonam may be due to efflux mechanism and increased production of cephalosporinases.

In 1996, VIM-2 was isolated in France from a *P. aeruginosa* isolate. This isolate was resistant to most β lactams but they are susceptible to aztreonam. VIM-2 is identical with 90% amino acid of VIM-1. The VIM family at present consists of 14 members and VIM-2 is the most-reported metallo β lactamase worldwide[34].

M.J. Carvalho et al in a study, 27 clinical isolates of *Pseudomonas aeruginosa* were found to be resistant to Imipenem and the PCR detected the presence of *bla*IMP and *bla*VIM gene[35].

In a study on molecular characterization of carbapenem resistant strains by Masoumeh Doosti, et al with 70 nosocomial isolates, forty four were resistant to Imipenem and DNA sequencing revealed the VIM-2 determinant[36].

Fereshteh Shahcheraghi et al in their study stated that out of 610 *Pseudomonas aeruginosa* isolates, 68 were found to be imipenem resistant with MIC ≥4 μg/ml. The PCR results for these 68 isolates showed only 16 isolates harbored *bla*VIM gene. No other genes like IMP, SPM were detected in this study[37].

Mariana Castanheira et al (2009) in their study isolated 301 *P.aeruginosa* collected from 10 Indian hospitals. The isolates producing VIM gene were detected in most of the participating hospitals and 5 *bla* VIM variants were detected[38].

Horieh Saderi et al in a study reported that out of 100 *Pseudomonas aeruginosa* isolates in burn patients, 65% isolates were found to be imipenem resistant and 13 among these isolates when subjected to PCR results in detection of VIM 2 gene. MBL genes like IMP were not detectable[39].

M. Shanthi Amudhan et al in her study concluded that the *bla*VIM gene was the most common MBL gene isolated. From a total of 179 samples, 61carbapenem resistant *Pseudomonas aeruginosa* were isolated.

About 51.4% isolates were positive for the presence of *bla*VIM and *bla*IMP and concluded that the VIM gene was more prevalent[40].

3.15.2.3.2.3 SPM (Sao Paulo MBL):

This was isolated from a *P. aeruginosa* isolate in 1997 in Sao Paulo in Brazil. The strains were found to be resistant to all antibiotics against gram-negative bacteria except Polymixin E. This is more often related to IMP gene.

Alexandre R. Marra et al 2005, from Sau Paulo Brazil, reported that out of the 76 *P. aeruginosa* isolates, four(5.3%) of them showed MBL gene *bla*SPM-1 recovered from the blood stream infections and the other genes obtained were *bla*IMP [3.9%] and no *bla*VIM MBL gene was detected[41].

Maria Renata Gomes Franco et al in their study reported that about 30.4% of the carbapenem resistant *P. aeruginosa* isolates were MBL positive, with 81% positive for SPM-1 and 19% positive for VIM-2. They also concluded that SPM-1 (Sao Paulo) metallo-b-lactamase is the most prevalent MBL in Brazil[42].

3.15.2.3.2.4 GIM-1 (German imipenemase) [26]:

In Germany, a newer β-lactamase named GIM-1 was isolated from five *P. aeruginosa* isolates in 2002. These isolates were susceptible only to polymyxin B.

GIM-1 has two zinc ions at its active site is the main feature of the MBL class B1 family. GIM-1 is similar to that of IMP-1.

3.15.2.3.2.5 SIM-1 ("Seoul imipenemase") [25]:

The newer family of acquired metallo-β-lactamases was discovered from Korea. This SIM-1 enzyme has about 64 to 69% amino acid identity to the IMP family. SIM-1 metallo -β-lactamase is located within a class 1 integron.

The SPM, GIM, and SIM metallo-β-lactamases have not spread beyond their countries of origin. But, VIM and IMP genes are detected worldwide. These two particular genes are currently spreading into the *Enterobacteriaceae* beyond the *P. aeruginosa.*

In two different studies Bashir et al [8]., 2011, Pitout et al [29]., 2007 concluded that MBL genes have spread from *P.aeruginosa* to Enterobacteriaceae in recent years.

3.15.2.3.2.6 NDM-1(New Delhi Metallo β lactamase)

Recently a newer type of carbapenemases, New Delhi metallo β lactamase-1(NDM-1) is making a spurt all over the world. It was first detected in *Klebisella pneumonia* isolate from a Swedish patient of Indian origin in 2008. The gene was found in an integron. NDM-1 mimics little with other MBLs mainly with VIM. It possess unique residues near the active site, NDM- 1 also has an extra insert between locations 162 and 166, which is not present in other MBLs. The molecular mass of NDM-1 is 28 kD and is monomeric. It hydrolyzes all the beta lactams except aztreonam[43].

Refath Farzana et al in Bangaladesh in their study reported that out of 31 MBL positive isolates by PCR, 8 isolates were positive for NDM gene[44].

3.16 MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA:* (MDRPA)

The *P. aeruginosa* resistant to three or more antimicrobial classes are said to be multidrug resistant *P. aeruginosa* [45, 46]. The projected annual incidence of MDRPA infected patients have been increased from 2007 to 2010[47]. The rates of multidrug resistance increased from 4 to 14% from the year 1993 to 2002, with decreasing susceptibility to most of the aminoglycosides, beta lactams and ciprofloxacin[45].

Elizabeth et al in their studies reported that the multidrug resistant *P. aeruginosa* isolates in the blood stream are about 10-17% from 2005 to 2007[6].

3.17 PHENOTYPIC METHODS FOR MBL DETECTION:

Metallo beta lactamases (MBLs) produced by *Pseudomnonas aeruginosa* poses a serious problem due to their ability of transmission between the same as well as different bacterial species. Though there are no CLSI guidelines in the detection of MBL there are many different methods to detect MBLs which may be performed in the laboratory.

3.17.1 Meropenem – EDTA combined disc test (CDT):

Test organisms in 0.5 Mc Farland turbidity standard is inoculated on to Mueller Hinton agar plate as for the routine disc diffusion procedure.

Meropenem discs are used as per the CLSI guidelines. Two 10 μg Meropenem discs (Himedia) are placed on the plate. To one of the discs, 10 μL of 0.5 M EDTA solution is added. The plate is incubated aerobically at 35°C for 16 to 18 hours. The zone of inhibition of the Meropenem and Meropenem-EDTA discs are compared. In the CDT, if the zone of inhibition of the Meropenem with the EDTA disc is ≥ 7 mm than the Meropenem disc alone, then it is considered as MBL positive.

Valenza G, et al in 2010, with 489 clinical isolates when investigated for MBL production from Germany university hospital suggested that the CDT is a valid alternative to the molecular investigation of MBL detection[48].

Deeba Bashir et al from Kashmir in 2008 in their study regarding detection of MBL in clinical isolates reported that out of 283 *P. aeruginosa* isolates 38 were resistant to imipenem and 33 were found to be MBL producers by CDT and all of them showed reduction in MIC with E test and agar dilution method. This study suggested that due to the cost constraints of the E test and laborious procedure of the agar dilution method, a simple screening test like the CDT can be used[8].

Vasanthi et al in their study reported that the CDT and DDST were the two phenotypic tests that can be used for the detection of the MBL production[49].

A study by P Pandya et al 2011, on evaluation of phenotypic methods on MBL detection in gram negative bacilli reported that out of 450 *P. aeruginosa* isolates about 27 were positive for MBL production. The production of MBL was highest in *Pseudomonas* (9.92%) than other gram negative bacilli. The CDT detected 26 isolates (96.30%) and DDST detected 22 isolates (81.48%) as MBL producer and the author concluded that the CDT is the most sensitive method for detection of MBL production in gram negative bacilli and there is subjective variation in interpreting the result of DDST. The results were more obviously interpreted in CDT than DDST[50].

3.17.2 Meropenam-EDTA double disk synergy test (DDST):

The test strain is inoculated on to the Mueller Hinton agar plate. A Meropenem (10 μg) disc was placed 10 mm centre to centre from a blank disc containing 10 μL of 0.5 M EDTA (750 μg)[51]. The zone of inhibition in the area between Meropenem and the EDTA disc is enhanced in comparison with the zone of inhibition on the far side of the drug is interpreted as a positive.

Kumar SH, et al reported in their study that 26.9% of carbapenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species were detected as MBL by DDST and the author also stated that Meropenem was considered to be the best screening and confirmatory agent for detection of MBL and the gene more prevalent in that part was *bla*VIM [52].

In a study by Kalantar E, et al the DDST for the detection of MBL showed that out of the 100 *P. aeruginosa* isolates, 22 (22%) were positive for MBL [15].

Yousefi S, et al from Iran in his study stated that among 104 clinical isolates of *Pseudomonas aeruginosa,*39 (37.50%) were MBL positive by DDST[53].

Bogiel T, et al in his study reported that from 67 carbapenam resistant strains from intensive care units, about 72.9% of the isolates are positive for MBL by DDST.[54]

Johann D.D Pitout et al., 2005 stated that the DDST has 100% sensitivity and 97% specificity in detecting MBL production in *P.aeruginosa* and suggested that it is better than the MBL E test[29].

3.17.3 MBL E-test:

The MBL E-Test strip contains a dual sided dilution range of Meropenam (MP) (0.125 to 8μg/mL) and Meropenem with EDTA (MPI) (0.032 to 2μg/mL) has been reported to be the most sensitive format for MBL detection. The E-test was done according to manufacturer's instructions. MIC ratio of MP (Meropenem)/MPI (Meropenem-EDTA) of >8 or deformation of ellipse or phantom zone is considered to be positive for MBL production.

Kaleem F, et al in a study reported that the MBL E test can be used to confirm the MBL[55]. A study by van der Bij et al reported that the sensitivity

and the specificity for the E test was 100% and 95% respectively in the detection of MBL[56].

Saha R et al in their study reported that out of 87 isolates of *P.aeruginosa* 31 isolates were resistant to carbapenem and 61% of these 31 isolates, were MBL producers by CDT, while 48% were detected by E-test method[57].

3.18 INDIAN SCENARIO:

Various studies from India quoted the prevalence of MBL varying from 8-14% (Bashir et al[8]., 2011; Varaiya et al[58]., 2008; Hirakata et al[59]., 1998). Ram Gopalakrishnan et al in his study on a retrospective review of surveillance over the period 2001-2008 reported the antimicrobial resistance patterns in Indian hospitals have a high prevalence that up to 40% of *P. aeruginosa* was resistant to carbapenems[60].

Prashant Durwas Peshattiwar et al from Bijapur 2010, in his study reported that about 7.8% of *P.aeruginosa* was MBL producers[61].

Deeba Bashir et al from Kashmir in his study stated that the prevalence of MBL producing *P.aeruginosa* were about 11.66%[8]. Agrawal et al from Nagpur concluded that about 8.05 % of the *P.aeruginosa* produces the metallo beta lactamase enzyme[62]. In a study by Seema Bose et al from Maharashtra in their study reported that 15.71% were MBL producers[63].

There are some studies that showed higher prevalence rates in India. Vikas kumar et al revealed in his study from North India that 87.17% were metallo beta -lactamase producers [64]. Noyal et al from Pondicherry reported 50% of the resistant isolates were MBL producers[65]. The prevalence rate of MBL in *P.aeruginosa* isolates was 51.4% reported by Shanthi amudhan et al[40] from Chennai.

3.19 GLOBAL SCENARIO:

Various studies across the world have shown different rates of MBL production in *P.aeruginosa* isolates. The prevalence rate ranges from 43% - 82% in countries like Bangaladesh(Nasrin et al[66]), Brazil(Pellegrino et al[67]., Magalhaes et al[68].), Iran(H.Saderi et al[39].), Italy(Lagatolla et al[69]) and Korea(K.Lee et al[70]).

Ting-ting Qu et al reported the lower prevalence rate in hospitals of China as 9.1%[71]. Yoshichika Arakawa et al in their study stated that 4.4% of the *Pseudomonas aeruginosa* were MBL producers with IMP gene in Japan. The author also quoted that similar type of MBLs was also prevalent in Italy, U.K and Singapore[72]. In Asia, blaIMP and blaVIM are prevalent[40] as seen in the above studies and bla SPM were more prevalent in Brazil.

4. MATERIALS AND METHODS

In the present study 100 *Pseudomonas aeruginosa* isolates obtained from various clinical specimens like pus, urine, burn, wound, sputum, pleural fluid and CSF from inpatients admitted to Tirunelveli medical college, Tirunelveli were included. The study period was from April 2013 to May 2014.These isolates were studied for detection of MBL production in *Pseudomonas aeruginosa* including their antibiogram.

4.1 INCLUSION CRITERIA

1. Specimens from patients admitted in wards were only included.
2. All isolates of *Pseudomonas aeruginosa* confirmed by biochemical reactions
3. Isolates showing resistance to Meropenem were only tested for production of metallo beta lactamase enzyme.

4.2 EXCLUSION CRITERIA

1. Specimens from outpatient department.

4.3 METHODOLOGY

4.3.1 Collection and Processing Of Various Samples:

A total of 100 non replicate isolates of *Pseudomonas aeruginosa* from clinical samples were taken in the study. All samples were collected under aseptic precautions by standard procedures and processed according to standard guidelines. Brain heart infusion broth was used for the blood

culture. The broth which showed turbidity was sub-cultured onto the MacConkey Agar and blood Agar media using sterile technique.

The urine specimens were centrifuged and inoculated on to the culture media. The wound swab and the other specimens such as pus, sputum were inoculated in the culture media and was also used for direct gram stain. The incubation was done at 37˚C for a period of 18-24 hours aerobically.

4.3.2 Ethical clearance and Informed consent :

The ethical committee clearance was obtained from our institution and informed consent was obtained from all patients included in the study.

4.3.3 Proforma:

The proforma was filled with the details like name, age, sex, ward, clinical diagnosis, risk factors, undergone any surgery, duration of hospital stay and other parameters significant to the present study.

4.3.4 Sample storage:

The *Pseudomonas aeruginosa* isolates were sub-cultured on to nutrient agar slope and stored at 2 to 8˚C. The isolates were sub-cultured in every two weeks.

4.3.5 CULTURE IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA*:

The *Pseudomonas aeruginosa* isolates were identified by, morphology on Gram stained smear and morphology of colonies on solid

media like Nutrient agar, MacConkey agar and Blood agar, the characteristic odour in culture plates, oxidase test, motility, biochemical reactions and growth at 42˚C.

4.3.5.1 GRAM STAINING:

Pink colored bacilli arranged in discrete pattern were identified as Gram negative bacilli.

4.3.5.2 COLONY MORPHOLOGY:

Nutrient agar: smooth, large, green pigmented, translucent colonies with earthy odour.

MacConkey agar: Colourless, non-Lactose fermenting colonies with spreading edges.

Blood agar: Beta hemolytic, greyish white colonies with metallic sheen.

4.3.5.3 MOTILITY (HANGING DROP METHOD):

The active motility, characteristic of *P.aeruginosa* was seen.

4.3.5.4 BIOCHEMICAL REACTIONS:

The *P.aeruginosa* gave the following biochemical reactions,

- Positive catalase test
- Positive oxidase test
- Oxidative pattern in Hugh – Leifson Oxidation – Fermentation test
- Nitrate reduction to nitrite
- Indole not produced

- Citrate utilized
- Urea not hydrolyzed
- Alkaline / Alkaline with no gas in Triple sugar iron agar
- Methyl red negative
- Voges – Proskaeur negative

4.3.6 ANTIBIOTIC SUSCEPTIBILITY TESTING:

According to the CLSI guidelines the antibiotic susceptibility testing was done in the entire *Pseudomonas aeruginosa* isolates by Kirby Bauer disc diffusion method.

4.3.6.1 Kirby-Bauer's disc diffusion method:

About 3-5 colonies of the test organism were inoculated in 2 ml of peptone water and incubated for 2-4 hrs at 37˚C. Using Wickham's chart the turbidity of the inoculum was adjusted to 0.5 McFarland standards ($1.5x10^8$ CFU/ ml). A sterile cotton swab was soaked in the inoculum and a lawn culture was made on to the Muller-Hinton agar (MHA). By rotating the swab against the inner side of the test tube, excess broth was expressed. The panel of antibiotic discs was applied and incubated at 37˚C for 18-24 hours. The zone size was recorded and interpreted as per the CLSI guidelines 2013.

The three interpretive categories are described as follows.

Susceptible:

This indicates that the recommended antibiotic in appropriate dose for recommended period is the appropriate agent for treating the infection.

Intermediate:

This indicates that the tested organism may be inhibited by possible concentrations of certain drugs if higher concentrations of the drug can be used safely or if the infection includes a body site where that drug is physiologically concentrated (e.g., the urinary tract).

Resistant:

The antibiotic tested may not be an appropriate choice for the infection against the tested organisms either they are not inhibited by the concentration of the drug normally achievable with the recommended dose or because the test result vastly correlates with a resistance mechanism.

In this study the susceptibility of the organism was tested against following antimicrobials from Hi-media laboratories Ltd, Mumbai.

Table:1 Interpretation of Antibiotic susceptibility testing

Antibiotic	**Concentration in** μg	**Sensitive**	**Intermediate**	**Resistant**
Ceftriaxone	30	>23	20-22	<19
Cefotaxime	30	>26	23-25	<22
Ceftazidime	30	>18	15-17	<14
Gentamicin	10	>15	13-14	<12
Amikacin	30	>17	15-16	<14
Ciprofloxacin	5	>21	16-20	<15
Ofloxacin	5	>17	14-16	<13
Meropenem	10	>19	16-18	<15
Pip – Tazo	100	>21	15-20	<14

4.3.7 MBL detection by phenotypic methods:

4.3.7.1 Initial screening with Meropenem by disc diffusion method:

All the isolates of *Pseudomonas aeruginosa* are tested for the susceptibility for Meropenam by disc diffusion method as per the CLSI guidelines.

About 3-5 colonies from the 24 hour young culture is inoculated in the peptone water and incubated for 2-4 hours. 0.5 Mc Farland standards suspension was prepared by adjusting the turbidity and checked by placing the tube side by side against a white card containing several horizontal black lines. Using a sterile cotton swab the inoculum is inoculated on to the Muller Hinton agar by streaking evenly over the surface of the agar in three directions with the plate rotated approximately 60° for even distribution. The plate was dried for 3-10 minutes before applying the Meropenem disc. The sterile needle was used to apply the disc. The disc was placed 15mm from the edge of the plate. The plate was incubated at a temperature of 37˚C for 16-18 hours.

According to the CLSI guidelines the zone of inhibition was measured and interpreted. For Meropenem disc, zone size of ≥ 19mm was taken as sensitive while zone size of ≤ 15mm was taken as resistant.

4.3.7.2 Detection of MBL:

The isolates which were found to be resistant to Meropenem were selected. These strains are subjected to various phenotypic methods

like combined disc test (CDT), double disc synergy test (DDST) and MBL E Test using Meropenem and EDTA to detect MBL production. They are confirmed genotypically by RT-PCR. For the negative control, ATCC 27853 *Pseudomonas aeruginosa* was used.

4.3.7.2.1 Combined Disc Test (CDT):

Preparation of EDTA solution:

To prepare 0.5 M EDTA solution, 18.61g of disodium EDTA.2H2O was dissolved in 100 ml of distilled water. By using sodium hydroxide (NaOH) the pH was adjusted to 8.0. The solution was autoclaved. Every time for the procedure, 10 µl of 0.5 M EDTA solution was used.

CDT procedure:

As per the CLSI guidelines, the 24 hr young culture isolate of the test strain is inoculated on to the Muller Hinton agar plate. Two Meropenem discs 10 µg were placed on the dried agar plate. To one of the disc 10µl (750 µg) of 0.5 M EDTA solution was added. The plate was incubated at 37˚C aerobically for 16-18 hours. The zone of inhibition was measured for the Meropenem and the Meropenem EDTA combined disc. The increase in the diameter of the zone by more than or equal to 7mm (≥7mm) for the Meropenem EDTA combined disc was considered MBL positive when compared to Meropenem alone.

4.3.7.2.2 Meropenem-EDTA Double Disc Synergy Test (DDST):

The test strain was inoculated as lawn culture on to the Muller Hinton agar plate as per the CLSI guidelines. The Meropenem (10 µg) disc was placed 10 mm apart from the sterile blank disc. The sterile blank disc was added with 10 µl of EDTA and incubated at 37˚C for 16-18 hours. The enhancement of the zone of inhibition towards EDTA disc was considered positive for MBL production of the test strain.

4.3.7.2.3 MBL E Test:

MBL E test strip is a unique strip for the phenotypic detection of MBL is coated with mixture of Meropenem + EDTA and Meropenem in a concentration gradient manner. The strip is made of porous material and the antibiotics are distributed evenly on either side of the strip. It was used to determine the minimum inhibitory concentrations of the drug for the test strain. E test MBL strip has a double sided antibiotic concentration in a range of Meropenem (MP) 0.125 to 8 µg/ml and Meropenem + EDTA (MPI) 0.032 to 2 µg/ml with a fixed concentration of EDTA.

Procedure:

The inoculum was prepared with 4-5 colonies from 24 hr young culture and inoculated as a lawn culture on to the Muller Hinton agar after adjusting the turbidity to 0.5 Mc Farland standards. The MBL E test strip container was taken from the freezer and kept at room temperature for 15 minutes before opening .The strip was then taken with a sterile forceps or E

test applicator and applied to the dried agar surface with the MIC scale facing upwards. The plate was incubated aerobically for 16-18 hrs at 37^0C.

Interpretation:

The plate was read after the determined time of incubation. The MIC of the isolate was read where the zone of inhibition intersects the strip. The MIC for MP/MPI $\geq$ 8 or deformation of ellipse or phantom zone was considered positive for MBL production.

4.3.7.2.4 MBL detection by PCR:

The Meropenem resistant *Pseudomonas aeruginosa* isolates were further tested for *bla*IMP and *bla*VIM gene by Real-Time PCR. The PCR kit was procured from Helini Biomolecules, Chennai, India. According to the manufacturer's instructions, the procedure was performed.

Requirements:

- Micro Pipettes variable volume 0.5-10 µl, 10-100 µl, and 100-1000 µl
- Sterile tips.
- Vortex mixer
- Water bath
- 13,000 rpm Centrifuge (Refrigerated) with rotor for 1.5ml reaction tubes
- 1.5ml/2ml centrifuge tubes
- Thermo cycler (Biorad CFX 96)
- Computer for data storage

DNA Extraction:

The DNA extraction procedure yields purified DNA of more than 30kb in size obtained after the lysis of the cell. This kit utilizes the silica based membrane technology in the form of a spin column. The isolated DNA can be used directly for the PCR amplification.

Components of extraction

- Phosphate buffered saline
- Lysozyme
- Digestion buffer
- Binding buffer
- Proteinase K
- Internal control template
- Isopropanol
- 70% Ethanol
- Elution buffer

Storage and stability:

The bacterial genomic DNA extraction kit was stored at room temperature.

The proteinase K and Lysozyme were stored at -20 ^{0} C.

Bacterial pellet preparation:

About 4-6 colonies of the test strains were inoculated in peptone water and incubated at 37^0C overnight. 1-1.5 ml of bacterial culture was transferred into the sterile 2ml centrifuge tube. The tube was centrifuged at 8000rpm for 5 minutes at room temperature. The supernatant was discarded and the pellet was used for the DNA extraction.

Principle:

The cells are lysed with the enzyme Proteinase K and the nucleases are inactivated by chaotropic salt. The nucleic acids are bound to special silica fibres in the spin column tube. The cellular components contaminating the bound nucleic acid is removed in a series of rapid "wash and spin" steps. The elution releases the nucleic acids from the silica fibre.

DNA extraction procedure:

1. The bacterial pellet was suspended in 200 µl phosphate buffer saline and the suspension is vortexed briefly.
2. 180 µl of digestion buffer and 20 µl of lysozyme were added and vortexed for 10 seconds.
3. The mixture is incubated for 15 minutes at 37^0C.
4. 200 µl of binding buffer, 20 µl of proteinase K and 5 µl of internal control template was added and mixed well by pulse vortex. It was incubated in a water bath at 56^0C for 15 min.
5. 300 µl of Isopropanol is added and mixed well.

6. The entire sample was pipetted into the spin column. At 12,000rpm it was centrifuged for 1 minute. The flow through was discarded. The spin column was placed into the collection tube.
7. 500 µl of 70% ethanol is added to the spin column. At 12,000rpm it was centrifuged for 1 minute. The flow through was discarded. The spin column was placed into the collection tube. This step was done twice.
8. The empty spin column with the collection tube was centrifuged at 12,000rpm for 2 minutes to avoid residual ethanol.
9. The collection tube is discarded and the spin column was placed in to a fresh micro centrifuge tube.
10. 75 µl of the pre-warmed (warmed at 56^0C) Elution buffer was added to the centre of the spin column and incubated for 2 minutes at room temperature. It was centrifuged for 1 minute at 13,000rpm.
11. The spin column was discarded and eluted DNA was taken for the PCR procedure.

PCR Amplification:

***bla* IMP and *bla*VIM primer & probe mix:**

The *bla*IMP and *bla*VIM primer & probe mix consists of TaqMan probe which is florescent labeled with FAM, forward primer and reverse primer.

For VIM gene:

Forward primer:

CGCGGAGATTGAGAAGCAAA

Reverse primer:

AGCCGCCCGAAGGACATC

Probe sequence:

TTGGACTTCCTGTAACGCGTGCA

For IMP gene:

Forward Primer:

GGGCGTTGTTCCTAAACATGG

Reverse Primer:

CACGCTCCACAAACCAAGTG

Probe:

TGGTGGTTCTTGTAAATGCTGAGGC

Internal Control primer & probe Mix

The internal control primer & probe mix consists of TaqMan probe which is florescent labeled with HEX, forward primer and reverse primer. The internal control is included to make sure that PCR inhibitors removed during the DNA extraction procedure and the performance of PCR mix ingredients are good. The internal control if amplified shows that PCR inhibitors are not present in the sample and the nucleic acid purification is optimum. It helps to rule out false negative results.

Positive control:

*bla*VIM positive template is used as positive control. A positive result indicates that the primers and probes worked correctly in that particular experiment.

Negative control:

Nuclease free water as used as a negative control. A negative result indicates that there is no contamination in the reagents.

Materials required:

- Real time PCR machine with FAM/JOE channel
- 0.2ml PCR tubes/8well strips
- Micropipette and tips

PCR detection mix:

The *bla*IMP and *bla*VIM reaction mix for the samples consisted of probe PCR master mix 10µl, *bla* IMP primer probe mix 10µl, *bla*VIM and internal control primer probe mix 10µl, purified DNA sample 5µl and a total volume of 25µl.

For positive control mix, 5µl of positive control template was added instead of sample DNA and for negative control mix, 5µl of nuclease free water was added instead of sample DNA.

The negative control is added first followed by samples and finally positive control was added to prevent cross contamination.

The PCR detection mix was centrifuged after adding all the ingredients and they were placed in the thermo cycler where the PCR reaction was allowed to occur.

Table:2. *bla*IMP reaction mix for samples

S.No	**Components**	**Volume**
1.	Probe PCR Master Mix	10µl
2.	*bla* IMP Primer Probe Mix	10µl
3.	Purified DNA sample	5µl
4.	Total reaction volume	25µl

Table:3. *bla*VIM reaction mix for samples

S.No	**Components**	**Volume**
1.	Probe PCR Master Mix	10µl
2.	*bla*VIM/IC Primer Probe Mix	10µl
3.	Purified DNA sample	5µl
4.	Total reaction volume	25µl

PCR amplification steps:

Initial Denaturation:

This is the first step in the amplification procedure. The thermocycler raises the temperature to 95˚C for five minutes for Taq enzyme activation.

Denaturation:

The temperature is elevated to 95˚C for 20 seconds. The double stranded template DNA gets separated into two complementary strands.

Annealing:

When the temperature is decreased to 55˚C for 20 seconds the complementary binding of the two specific oligonucleotide primers to the DNA template take place.

Extension:

The DNA polymerase extends the primers when the temperature is increased to 72˚C for 20 seconds. The template DNA is synthesised using deoxynucleotides (dNTPs) in the reaction mixture. Two single stranded DNA templates and newly synthesized complementary DNA strands attach together to form new double stranded DNA copies. Every copy of newly formed DNA may function as a template for further amplification. The products will be amplified in an exponential manner in each cycle. At the end of 40 cycles, the final PCR products will have 2n copies of template DNA. Data analysis was made at the end of extension and the computer produces the cross threshold (Ct) value by calculating the fluorescence emitted at the end of each cycle.

Table:4. Amplification profile for *bla*IMP and *bla*VIM gene:

	Step	**Time**	**Temp**
	Taq enzyme activation	5min	95^0 C
40cycles	Denaturation	20sec	95^0 C
	Annealing/ Data collection	20sec	55^0 C
	Extension	20sec	72^0 C

VIM/IMP = FAM channel

Internal control = HEX channel

Interpretation of the results:

Negative control:

The negative control reactions should not exhibit fluorescence growth curves that cross the threshold line.

Positive control:

Positive control reactions should produce positive results before 40 cycles.

Test sample positive:

When all the controls fulfill the quantified requirements, a test sample is measured positive. Any samples which exhibit fluorescence that cross the threshold line at or after 38 cycles should be retested.

Test sample negative:

When all controls fulfill the quantified requirements, a test sample is measured negative.

Internal control interpretation:

According to the protocols (assuming 100% extraction efficiency) the CT value is expected within 24-31 for the internal control. This may differ depending on the extraction efficiency, the quantity of elute added to the PCR reaction and the settings of machine. If the amplifying sample had high genome copy number then the internal control may not produce an amplification plot. This can be interpreted as positive result rather invalidates it.

Table:5. Interpretation of results

Test Sample	**Negative control**	**Internal control**	**Positive control**	**Interpretation**
Positive	Negative	Positive	Positive	**Positive**
Negative	Negative	Positive	Positive	**Negative**
Negative	Negative	Negative	Negative	**Repeat**
Positive	Positive	Positive	Positive	**Repeat**

FIG. 1. NUTRIENT AGAR PLATE SHOWING GREEN PIGMENTED COLONIES

FIG.2. MACKONKEY AGAR PLATE SHOWING NON-LACTOSE FERMENTING COLONIES

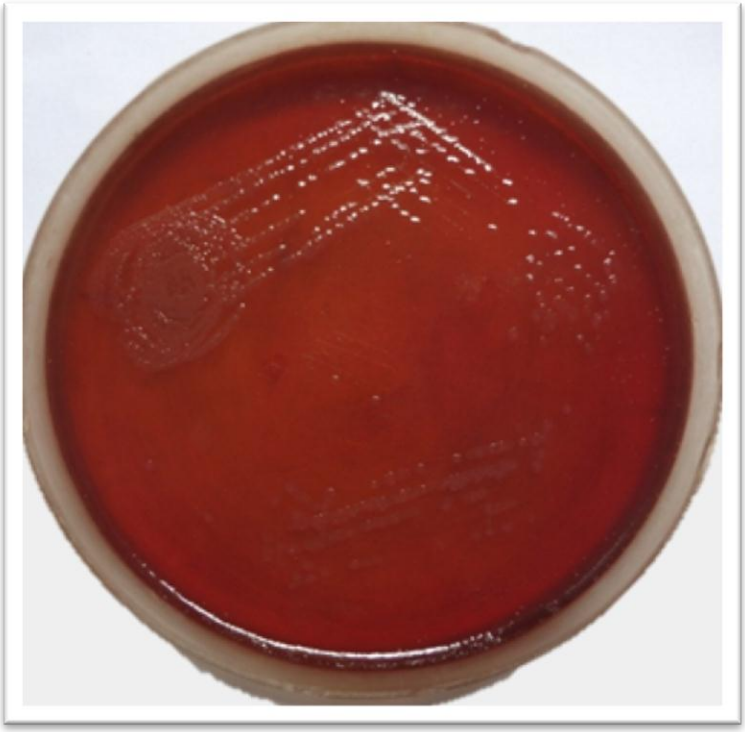


FIG.3. BLOOD AGAR PLATE SHOWING METALLIC SHEEN AND BETA HEMOLYTIC COLONIES

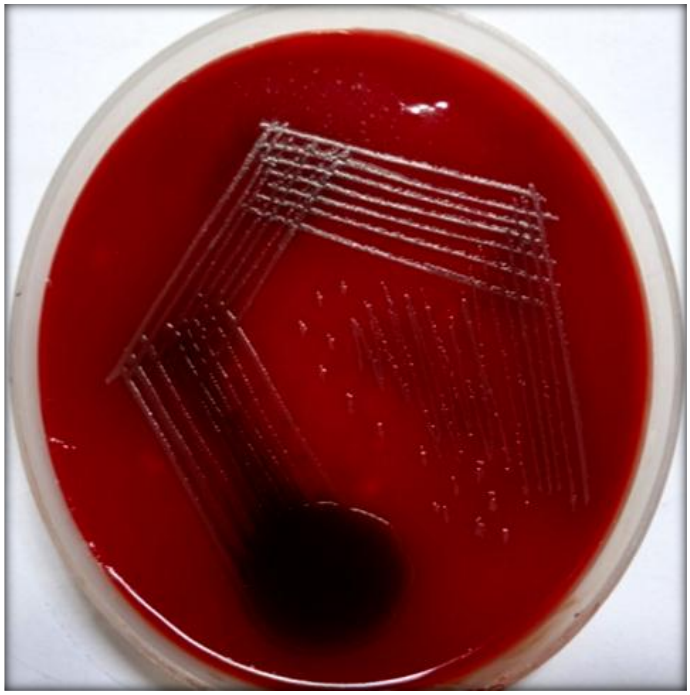


FIG. 4. OXIDASE TEST

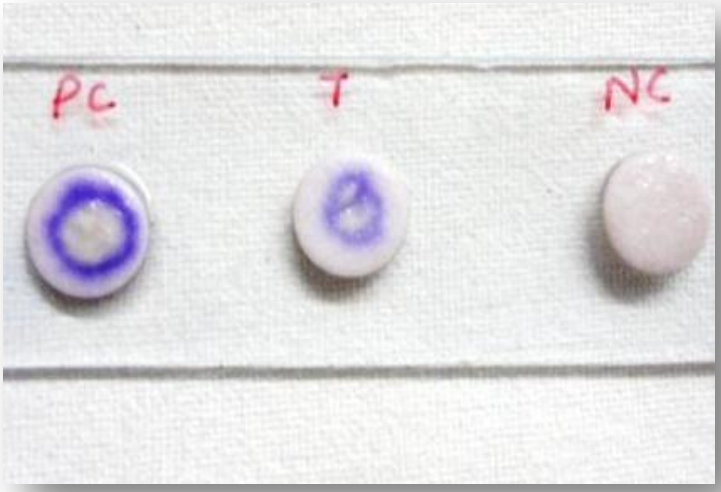
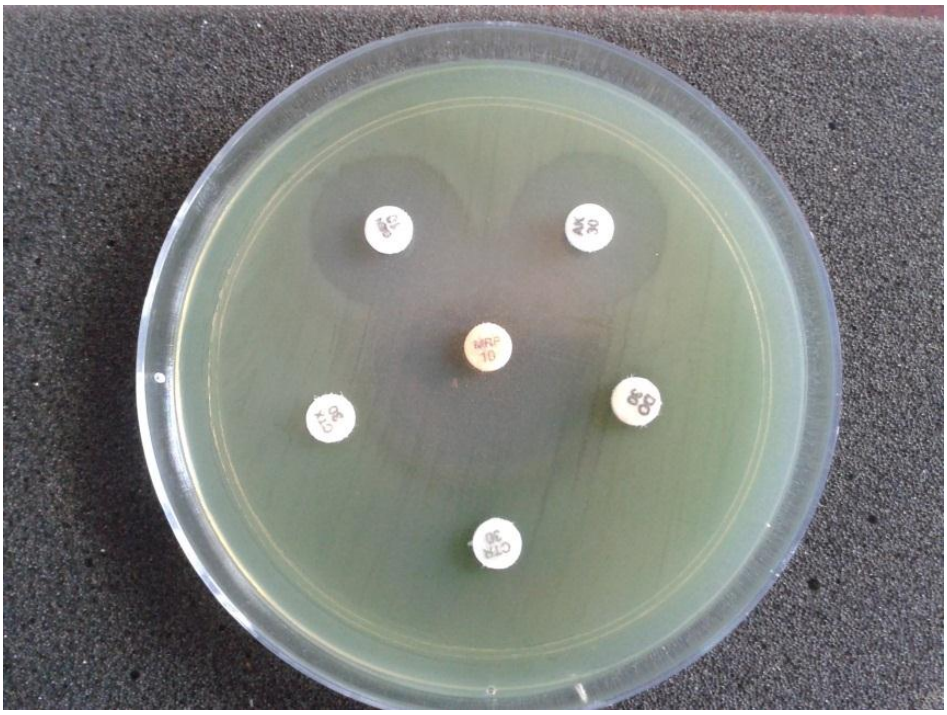


FIG.5. BIOCHEMICAL REACTIONS

FIG.6. Antibiotic susceptibility test



COMBINED DISC TEST

Fig:7. MBL Producer

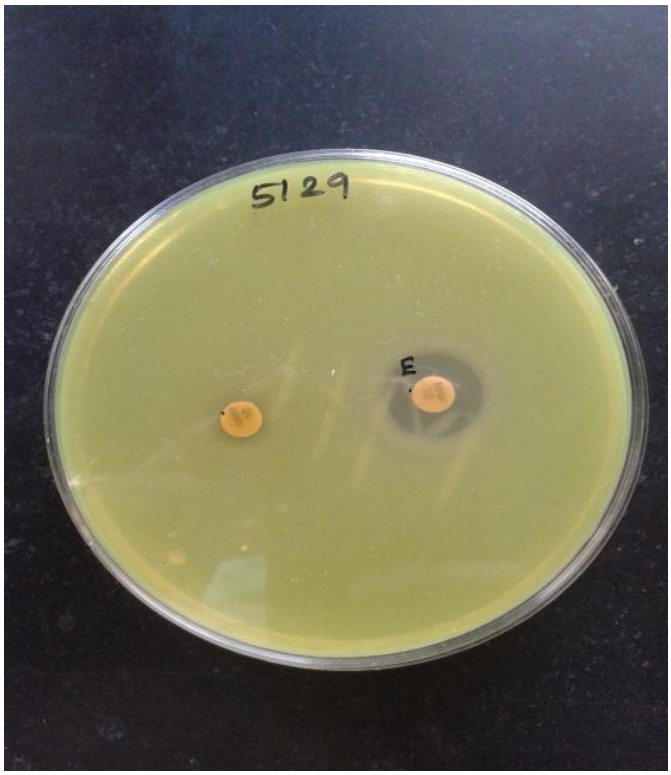
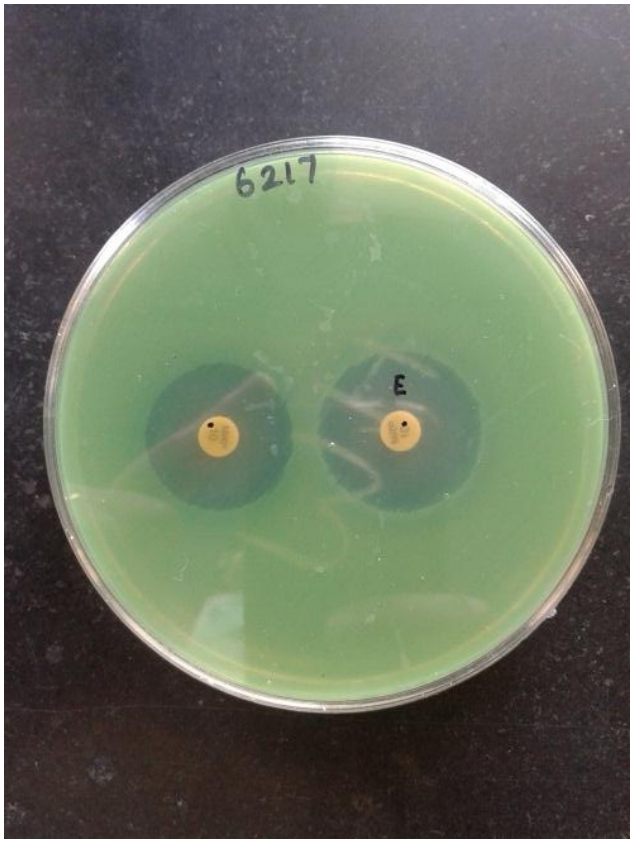


Fig:8. Non MBL Producer



DOUBLE DISC SYNERGY TEST
THE ENHANCEMENT OF THE ZONE OF INHIBITION FROM MEROPENEM DISC TOWARDS EDTA DISC

Fig:9. MBL producer by DDST

MBL E Test

Fig:10. MBL-PRODUCER:

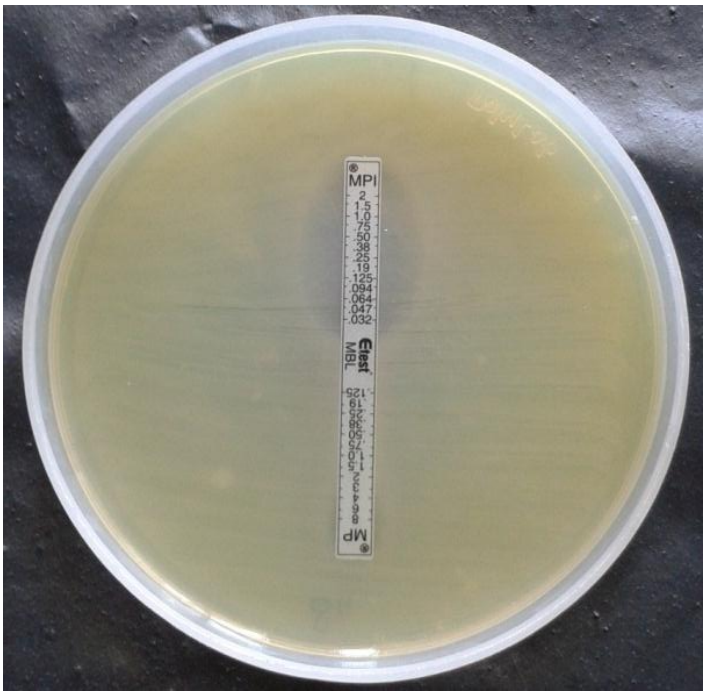


Fig:11. NON-MBL PRODUCER:

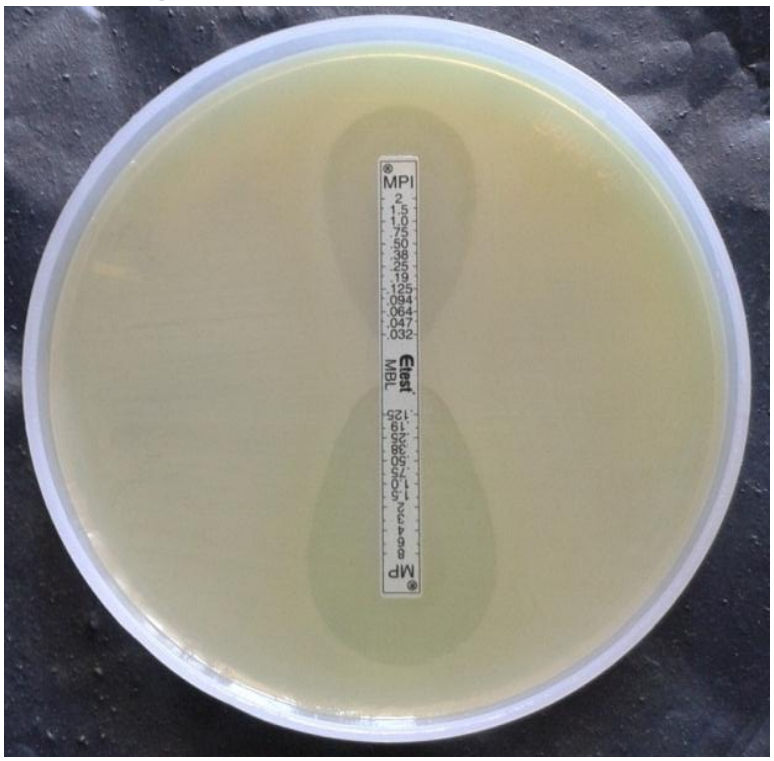


Fig:12. MOLECULAR CHARACTERISATION by PCR: THERMOCYCLER

Fig:13 DNA Extraction Kit:

Fig:14 DNA extraction:

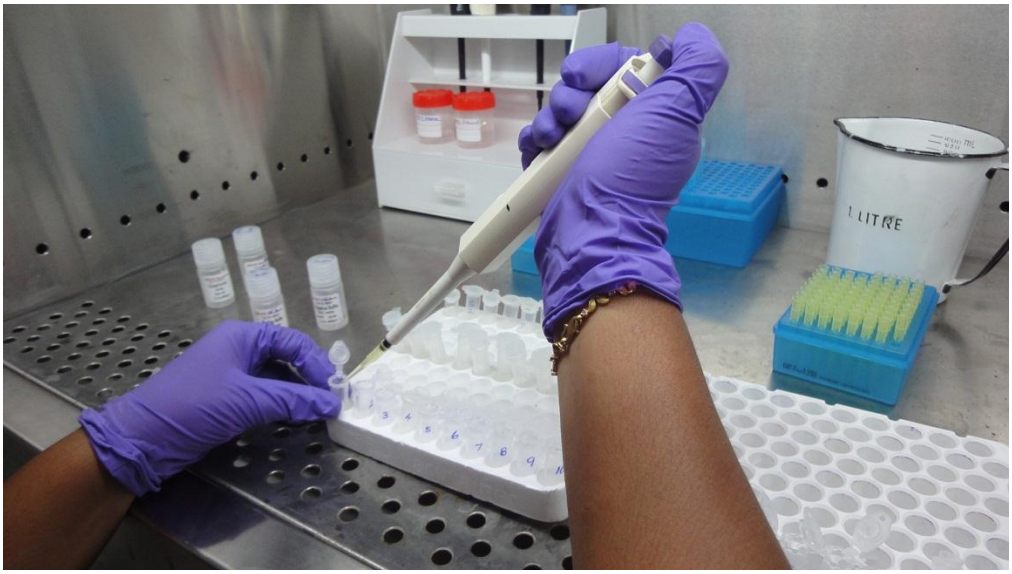


Fig:15. PCR Amplification Kit:

Fig:16. Loading samples in Thermocycler:

5. RESULTS

5.1 Study Description:

The study was conducted at the Department of Microbiology, Tirunelveli Medical College, over a period of one year from April 2013 to May 2014. A total of 100 *Pseudomonas aeruginosa* isolates from various clinical samples were included in the study. These isolates were subjected for Meropenem resistance by disc diffusion test and the resistant isolates were tested for MBL with phenotypic tests like CDT (Combined disc test), DDST (Double disc synergy test and MBL E test. They were also tested for the presence of genes like *bla*IMP and *bla*VIM by Real-Time PCR. The sensitivity patterns to the antibiotics for the isolates and the other risk factors were analysed.

5.2 Statistical Analysis:

Data regarding the subjects were defined in terms of percentages. The statistical measures were completed with the help of the statistical software IBM SPSS statistics 20. The p values < 0.05 was considered as significant ($p < 0.05$) in Chi square and Mc Nemer test.

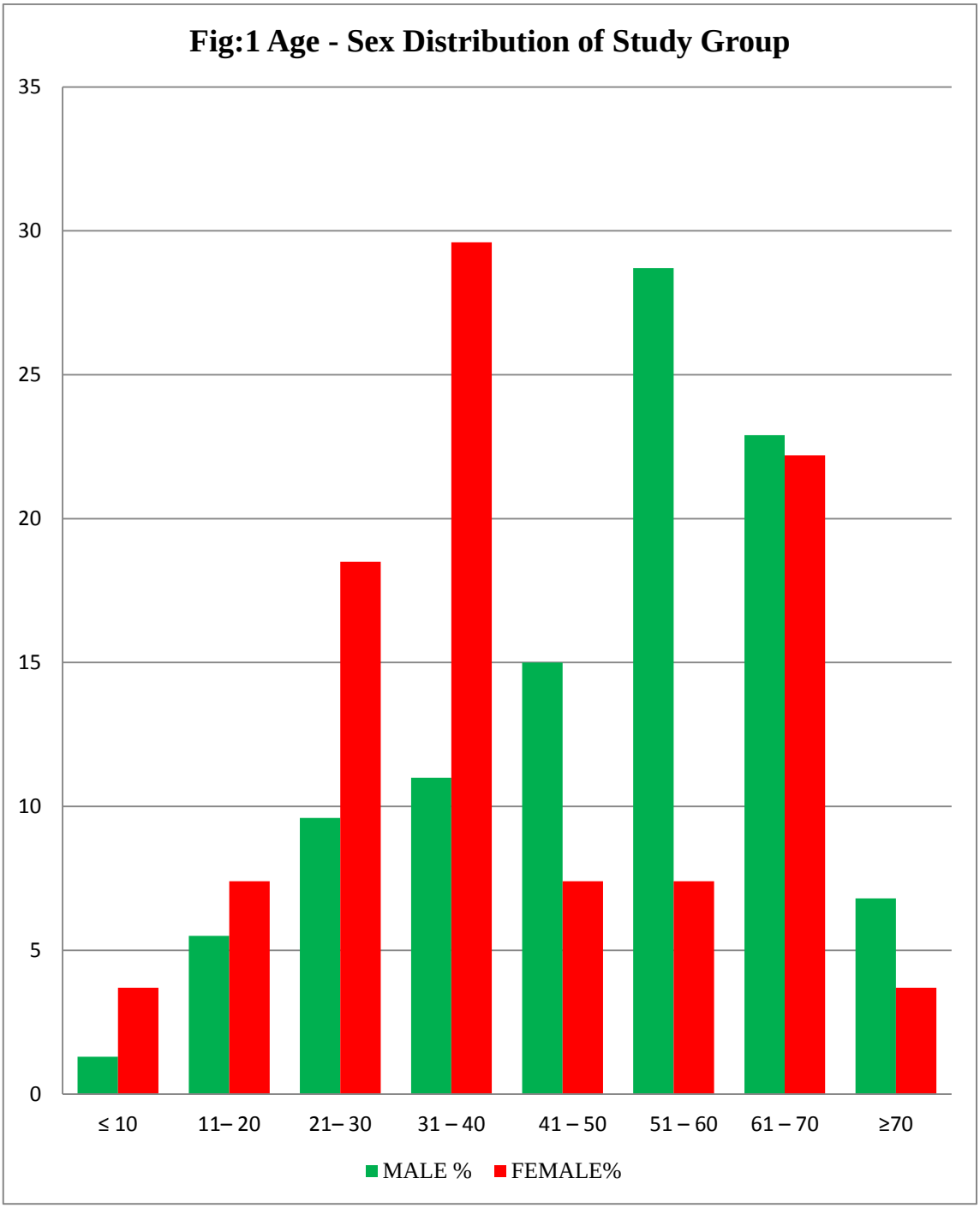
5.3 Analysis by age and gender:

Table:6 Age – Sex distribution of the Study group

Age (years)	Male		Female		Total	
	No	%	No	%	No	%
≤ 10	1	1.3	1	3.7	2	2
11– 20	4	5.5	2	7.4	6	6
21– 30	7	9.6	5	18.5	12	12
31 – 40	8	11	8	29.6	16	16
41 – 50	11	15	2	7.4	13	13
51 – 60	21	28.7	2	7.4	23	23
61 – 70	16	22.9	6	22.2	22	22
≥70	5	6.8	1	3.7	6	6
Total	73	73	27	27	100	100

Out of 100 isolates, 86 isolates were between the age group of 21 and 70 years. Among them 63 were males and 23 were females. The mean age of male was 50.75 years and that of female was 41.59 years. (Table: 6; Figure.1)

Fig:1 Age - Sex Distribution of Study Group



5.4 Distribution of *Pseudomonas aeruginosa in clinical isolates:*

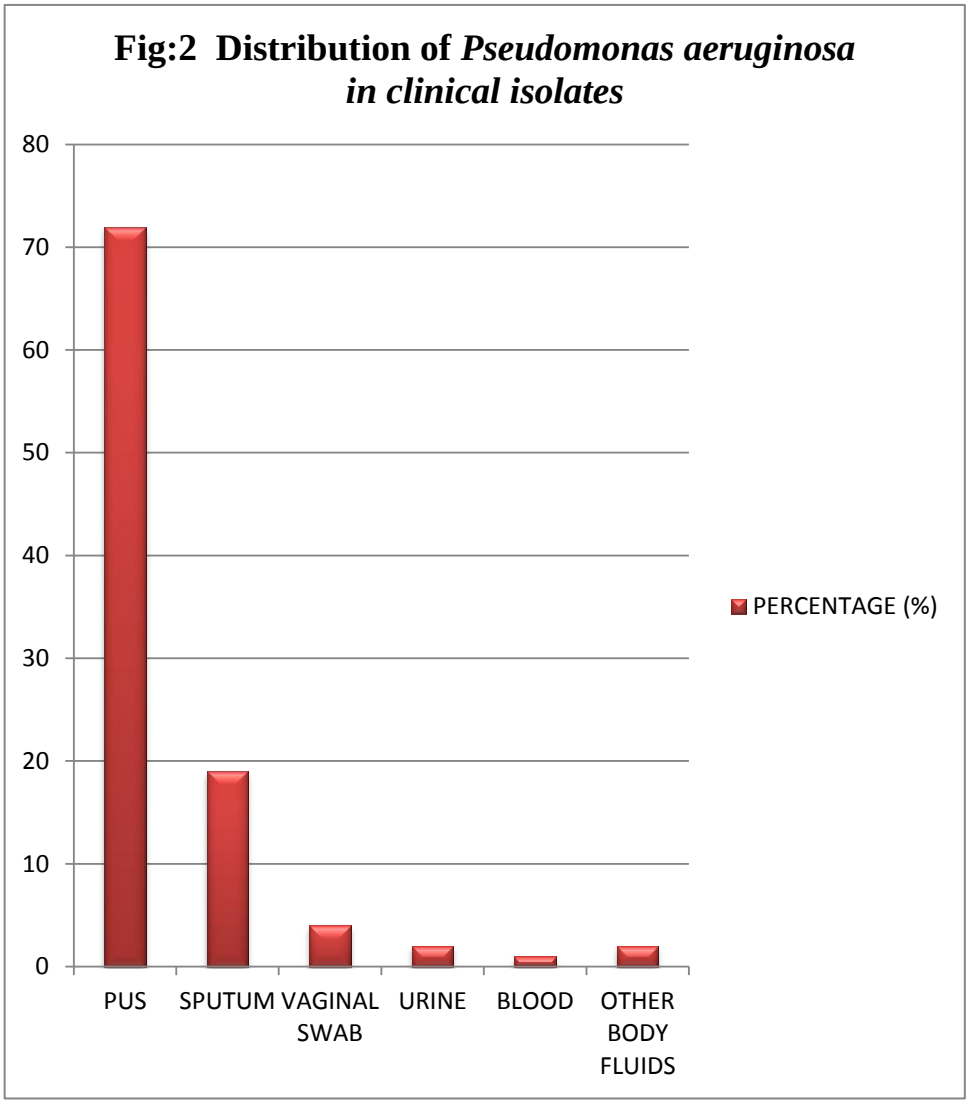
The specimen wise distribution of *Pseudomonas aeruginosa* was analysed and about 72 isolates were from pus samples. 19 and 4 samples were from sputum and vaginal swab respectively. Two isolates were from urine samples and only one isolate was obtained from blood. Two isolates were from other body fluids (CSF and pleural fluid). Fig: 2.

Table:7 Distribution of *Pseudomonas aeruginosa in clinical isolates:*

S.No	Specimen	Number	Percentage (%)
1	Pus	72	72
2	Sputum	19	19
3	Vaginal Swab	4	4
4	Urine	2	2
5	Blood	1	1
6	Other Body Fluids	2	2

Other body fluids: CSF, Pleural fluid.

Fig:2 Distribution of *Pseudomonas aeruginosa* in clinical isolates



5.5 Resistance patterns of *Pseudomonas aeruginosa* to antibiotics:

Table: 8 Resistance patterns to Antibiotics

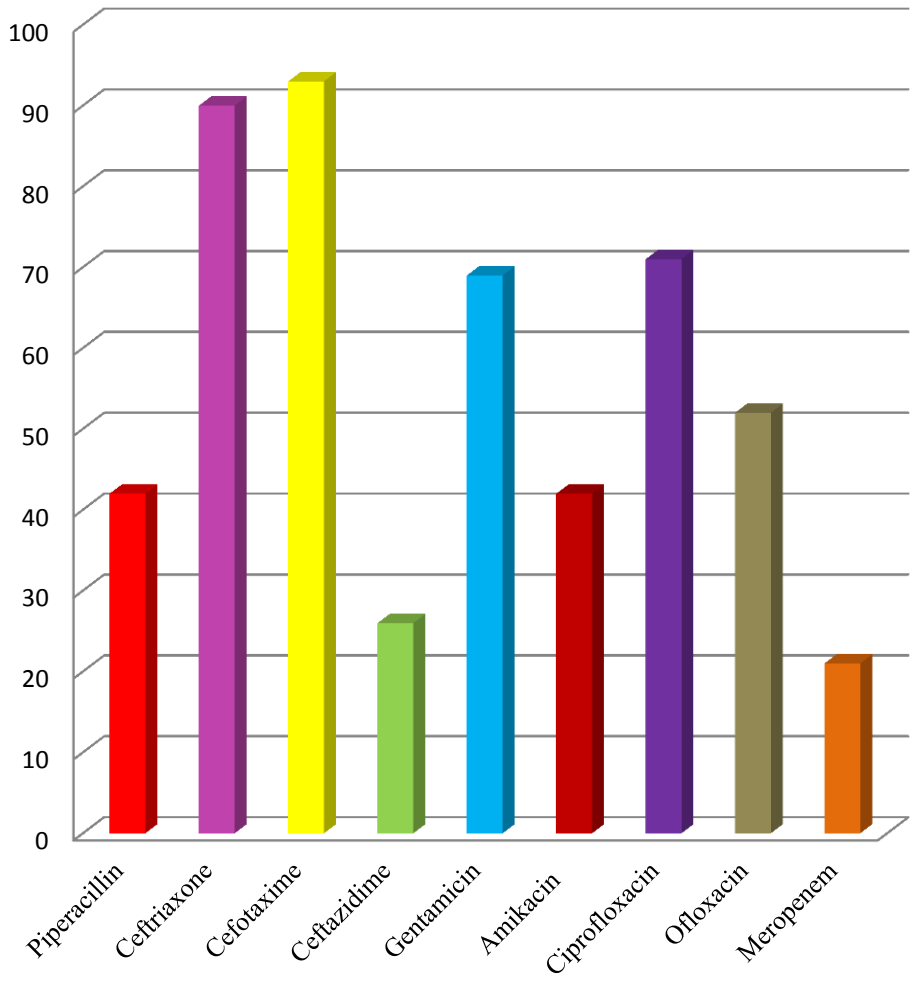
Antibiotic discs	No .of resistant Isolates	Percentage (%)
Gentamicin (GEN) 10 µg	69	69
Amikacin (AK)30 µg	42	42
Ciprofloxacin (CIP) 5 µg	71	71
Ofloxacin (OF) 5 µg	52	52
Ceftazidime (CAZ)30 µg	26	26
Ceftriaxone (CTR) 30 µg	90	90
Cefotaxime (CTX) 30 µg	93	93
Piperacillin 100 µg	42	42
Meropenem (MRP) 10 µg	21	21

The above table shows the resistance pattern of the various antibiotics to the *Pseudomonas aeruginosa*. 42% of the isolates show the resistant to Piperacillin. The resistant to the Ceftriaxone and cefotaxime is very high and it is more than 90%. The aminoglycosides like Gentamicin and Amikacin show resistance of 69% and 42% respectively. The Meropenem and Ceftazidime show about 21 and 26 percentages respectively. Among the

100 isolates, 21 were Meropenem resistant *Pseudomonas aeruginosa* (MRPA n=21) and remaining 79 were Meropenem sensitive *Pseudomonas aeruginosa* (MSPA)

Resistance patterns of *Pseudomonas aeruginosa* to antibiotics

Fig: 3 Resistance patterns of *Pseudomonas aeruginosa* to antibiotics



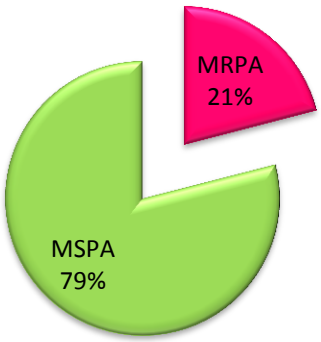
5.6 Screening test for MBL detection:

Table: 9 Meropenem Resistance in *Pseudomonas aeruginosa* isolates by Disc Diffusion test:

Total No of *Pseudomonas aeruginosa* isolates	Meropenem Resistant *Pseudomonas aeruginosa* (MRPA)	Meropenem Sensitive *Pseudomonas aeruginosa* (MSPA)
100	**21**	**79**

The above table shows the results of initial screening test in the detection of MBL determined by disc diffusion test using Meropenem (10µg). The zone of inhibition<15mm is taken as resistant. Among the entire *P.aeruginosa* isolates 21 isolates showed resistance to Meropenem.

Fig: 4 Meropenem Resistance in Pseudomonas aeruginosa isolates

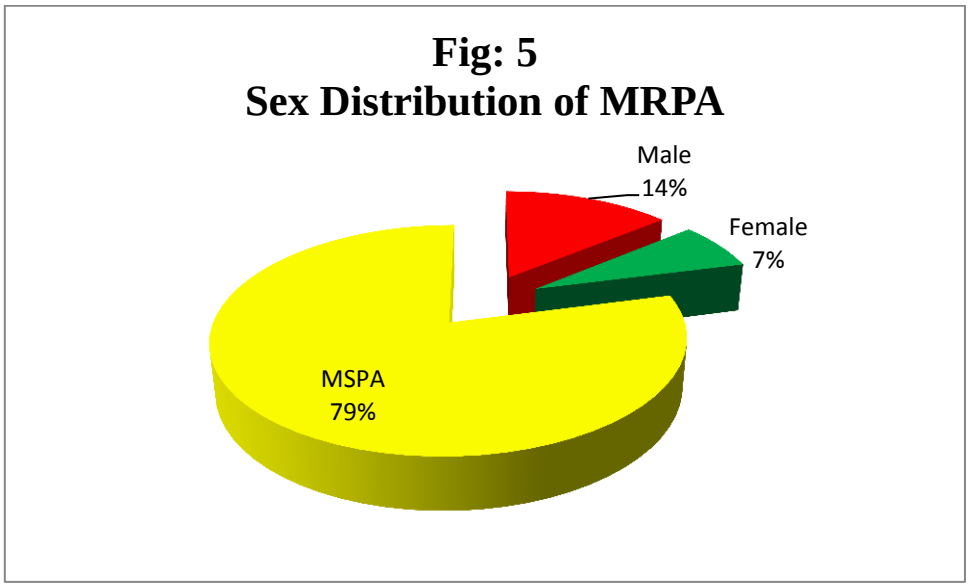


5.7 Sex-wise distribution of MRPA:

Table: 10 Sex-wise distribution of MRPA

Gender	MRPA	Percentage	MSPA	Percentage	Total
Male	14	19.2	59	80.8	73
Female	7	25.9	20	74.1	27

The above table describes the gender distribution in MRPA isolates. Among the 21 MRPA isolates, 14 were males and 7 were females. In case of MSPA about 59 isolates were from males and 20 from females. The p value with the chi square test is 0.462 which is not statistically significant. (Table:10, Fig:5)



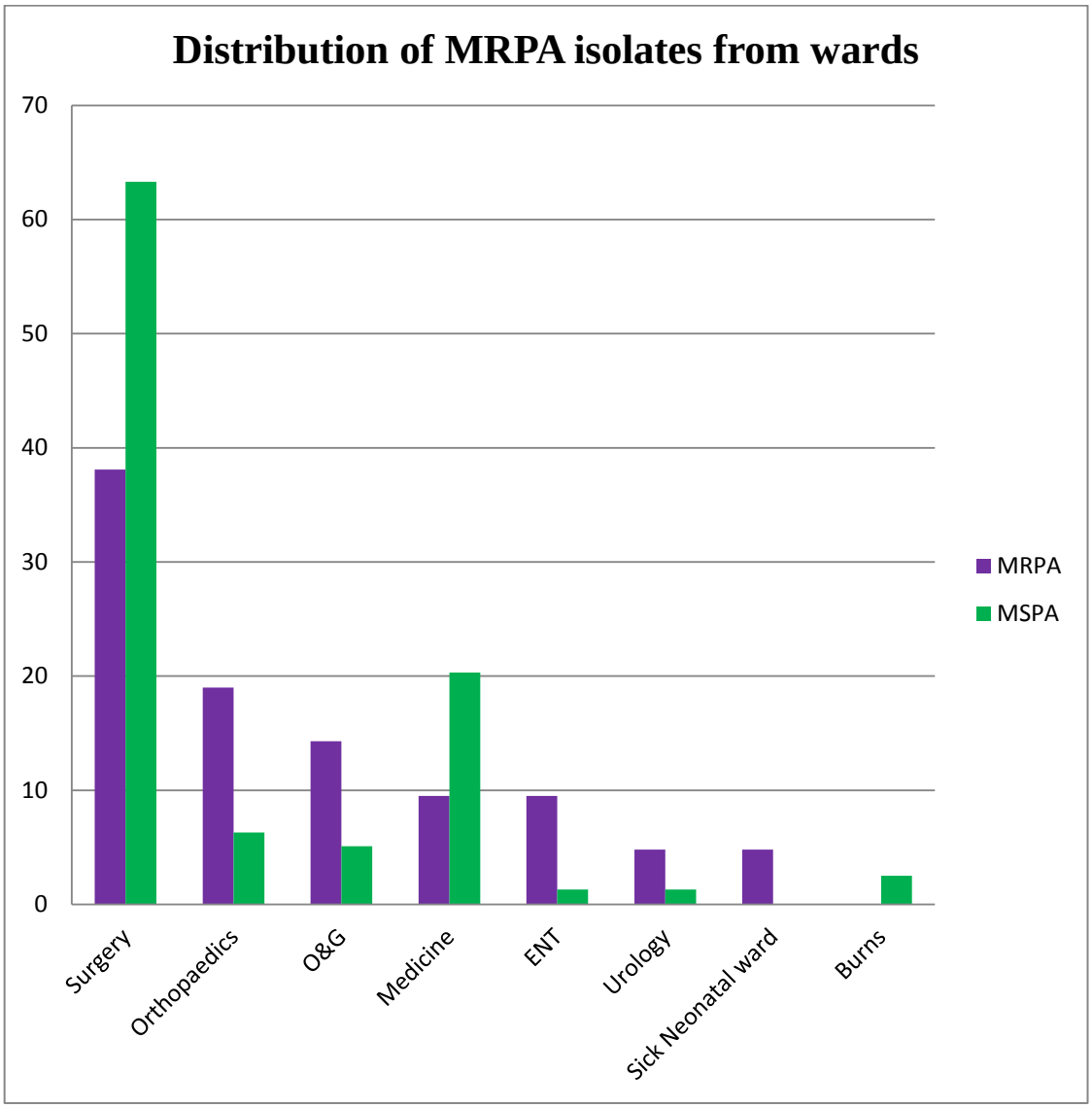
5.8. Distribution of MRPA isolates from various wards:

Table: 11 Distribution of MRPA isolates from wards

Ward	MRPA		MSPA	
	No	%	No	%
Surgery	8	38.1	50	63.3
Sick Neonatal ward	1	4.8	0	0
Orthopaedics	4	19.0	5	6.3
O&G	3	14.3	4	5.1
Burns	0	0	2	2.5
ENT	2	9.5	1	1.3
Urology	1	4.8	1	1.3
Medicine	2	9.5	16	20.3
Total	**21**	**100**	**79**	**100**

The above table shows the distribution of MRPA samples from various departments of the hospital. Majority of the MRPA isolates were from Surgery department i.e 8 (38.1%) from the total of the 58 isolates. About 4 MRPA isolates (19%) were from Orthopaedics department which contributes the second major cases. Three isolates were from O&G department and 2 isolates were from medicine and ENT department respectively. (Table:11, Fig:6)

Fig: 6 Isolation of *Pseudomonas aeruginosa* from wards



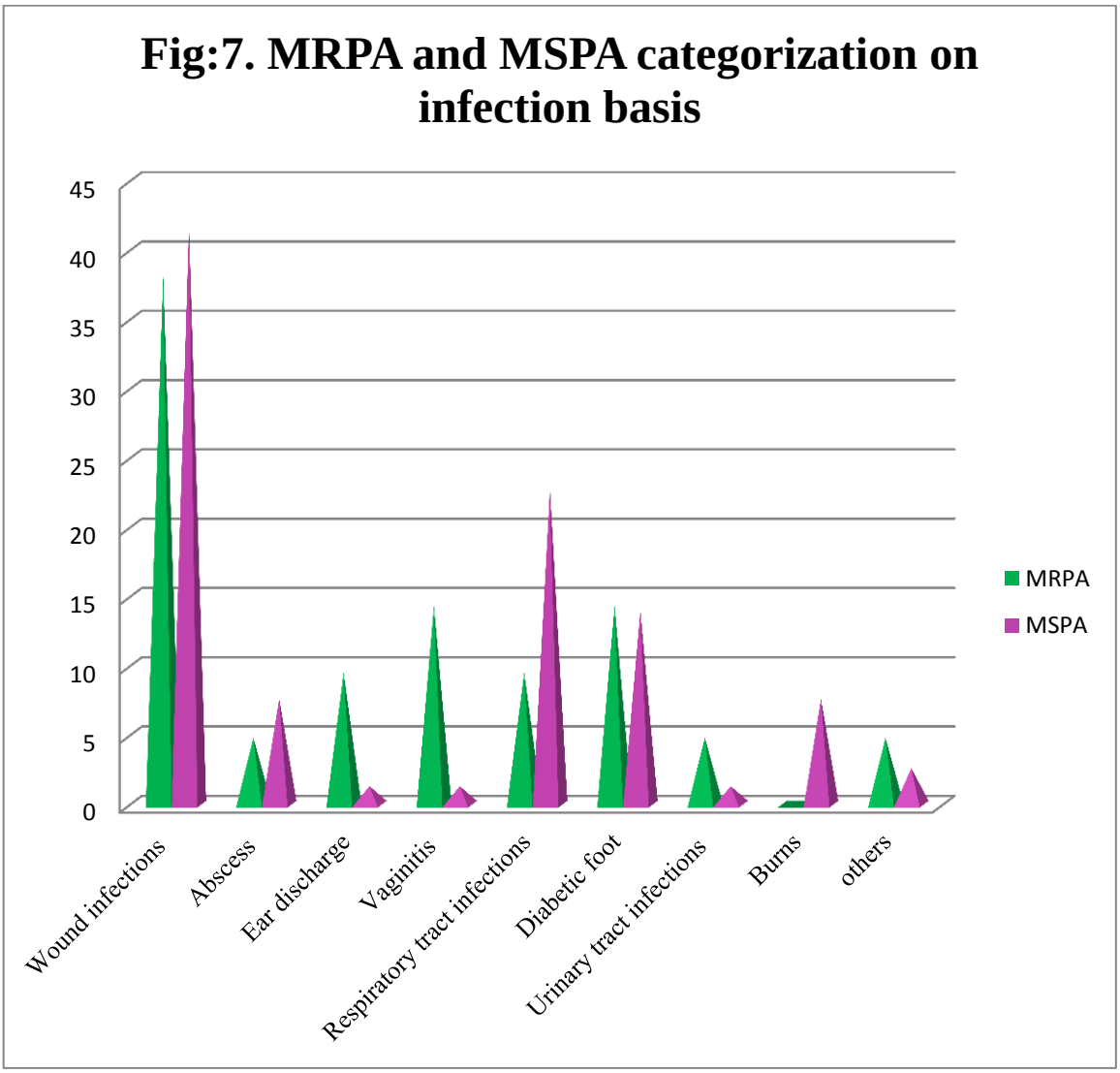
5.9. Categorization of MRPA and MSPA on infection basis:

Table: 12 Categorization of MRPA and MSPA on infection basis:

Infections	**MRPA**		**MSPA**	
	No	**%**	**No**	**%**
Wound infections	8	38.1	33	41.8
Abscess	1	4.8	6	7.6
Ear discharge	2	9.5	1	1.3
Vaginitis	3	14.3	1	1.3
Respiratory tract infections	2	9.5	18	22.8
Diabetic foot	3	14.3	11	14.0
Urinary tract infections	1	4.8	1	1.3
Burns	0	0	6	7.6
Others	1	4.8	2	2.6
Total	**21**	**100**	**79**	**100**

Table 12 shows the categorization of *Pseudomonas aeruginosa* on infection basis. On analyzing various infections associated with *Pseudomonas aeruginosa*, 38.1% of MRPA isolates were associated with

wound infections, 14.3% of MRPA isolates were associated with vaginal infections and diabetic foot, 9.5% constitutes respiratory infections and ear infections. (Fig.7)



5.10. Analysis of various phenotypic methods for the detection of the MBL production:

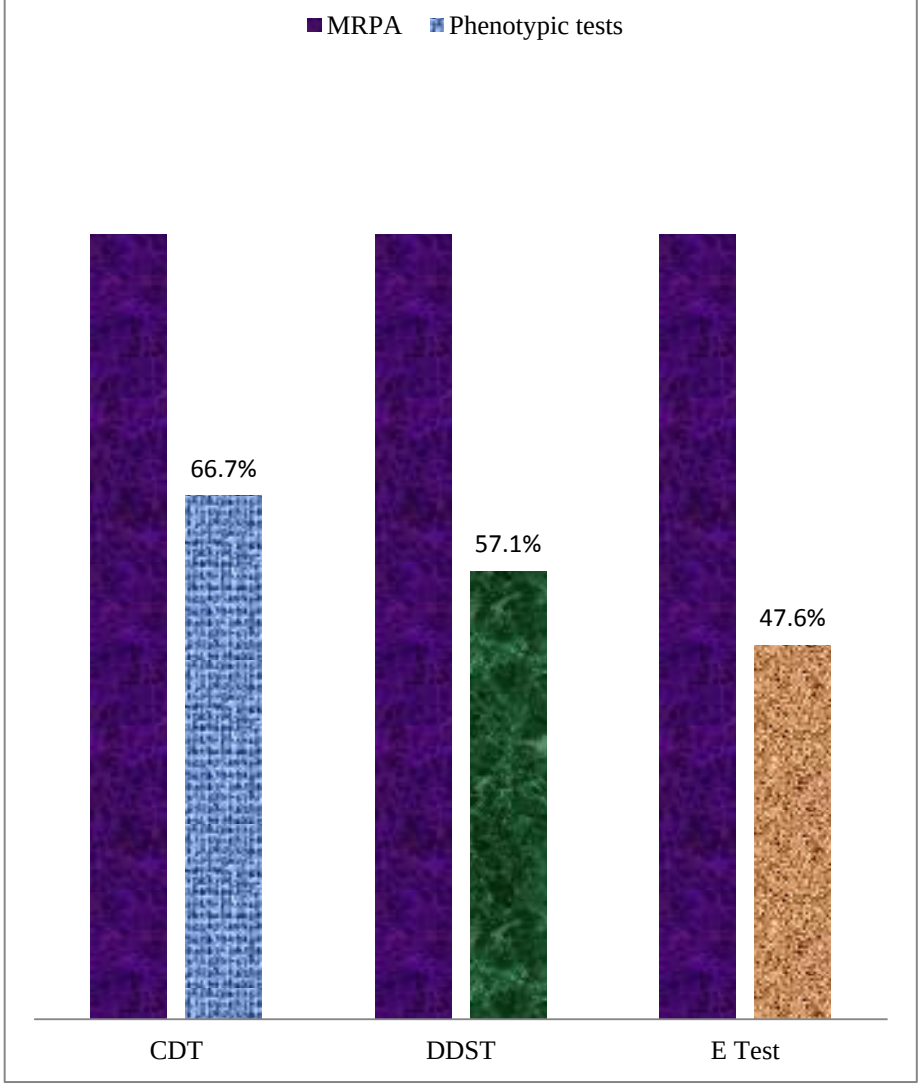
The 21 isolates which showed resistance to Meropenem were evaluated for the production of MBL by various phenotypic methods like CDT, DDST and MBL E test. Among them, 14 isolates showed MBL production by CDT and 12 were positive with the DDST. The MBL E test detected about 10 isolates which were considered positive for MBL production. (Table:13 and Fig:8)

Table: 13 Detection of MBL by various phenotypic methods

MRPA n = 21

Method	MBL producer	
	No	%
CDT	14	66.7
DDST	12	57.1
E Test	10	47.6

Fig: 8 Detection of MBL by Phenotypic methods

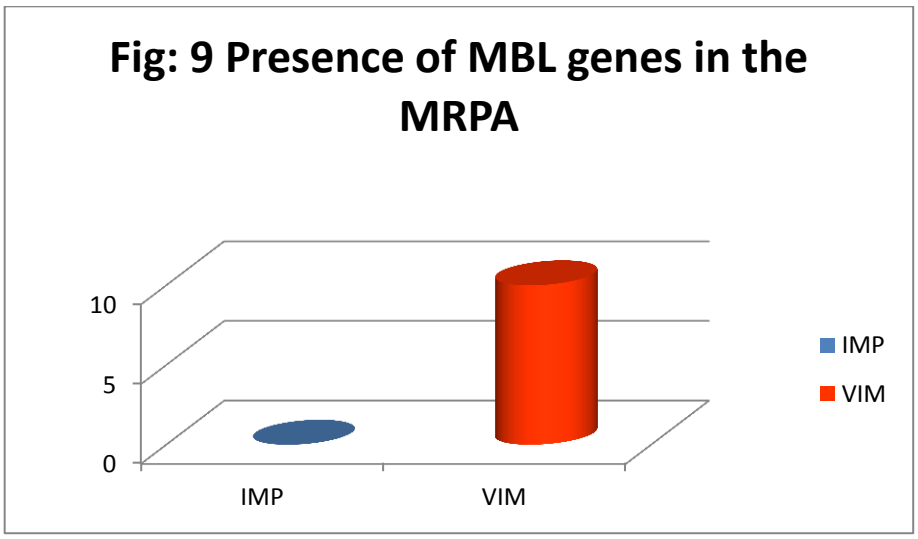


5.11. Prevalence of *bla*VIM and *bla*IMP gene in the Meropenem resistant isolates:

Table: 14. Detection of MBL by REAL TIME PCR.

Gene amplified	No of isolates	Percentage %
VIM	10	47.6
IMP	0	0
Not amplified	11	52.3
Total	21	100

Among the 21 Meropenem resistant isolates, ten (47.6 %) were positive for the presence of *bla*VIM gene by Real Time-PCR. *bla*IMP gene was not detected in any of the Meropenem resistant isolates. (Table 14, Fig:9).



5.12. Comparison of CDT and PCR for detection of MBL:

Table: 15. Comparison of CDT and PCR for detection of MBL

CDT	PCR		
	Positive	**Negative**	**Total**
Positive	9	5	14
Negative	1	6	7
Total	10	11	21

Among the 21 MRPA isolates, 14 isolates gave positive result with the CDT. The PCR detected the presence of MBL gene in 10 isolates. About 9 CDT positive isolates and one CDT negative isolate were PCR positive.

The sensitivity, specificity, positive predictive value and negative predictive value of CDT in the detection of MBL were 90%, 55%, 64.2% and 85.7% respectively. (Table: 15). The kappa value denoting the measure of agreement is low (0.438).

5.13. Comparison of DDST and PCR for detection of MBL:

Table: 16. Comparison of DDST and PCR for detection of MBL:

DDST	PCR		
	Positive	**Negative**	**Total**
Positive	9	3	12
Negative	1	8	9
Total	10	11	21

The DDST detected 12 among 21 MRPA isolates as MBL producer and the PCR detected the MBL gene in 10 MRPA isolates. Among 9 isolates that were negative with the DDST, one isolate was PCR positive and the remaining 8 isolates were also negative in PCR.

The sensitivity, specificity, positive predictive value and negative predictive value of CDT for detecting MBL were 90%, 72%, 90% and 61% respectively. (Table:16). The kappa value denoting the measure of agreement is moderate (0.622).

5.14. Comparison of MBL E test and PCR for detection of MBL:

Table: 17. Comparison of MBL E test and PCR for detection of MBL

MBL E Test	PCR		
	Positive	**Negative**	**Total**
Positive	9	1	10
Negative	1	10	11
Total	10	11	21

Among 21 MRPA isolates, 10 isolates were positive with the E test. The 9 isolates with E test positive and one isolate with E test negative was PCR positive.

The sensitivity, specificity, positive predictive value and negative predictive value of MBL E test were 90%, 90%, 90% and 90% respectively. (Table: 17). The kappa value denoting the measure of agreement is High (0.809).

5.15. Analysis of Risk Factors:

5.15.1. Duration of hospital stay:

About 81% of the MRPA isolates were obtained from the patients with more than 2 weeks of hospital stay. Only 20% of the MSPA isolates were from the patients with >2 weeks of hospital stay. The association of MRPA isolates with the duration of hospital stay was statistically significant. $P < 0.05$(Table.18 & fig.10)

Table: 18. MRPA isolates by duration of hospital stay

Duration in days	MRPA		MSPA	
	No	%	No	%
≤2 weeks	4	19.04	63	79.74
>2 weeks	17	80.96	16	20.26
Total	**21**	**100**	**79**	**100**

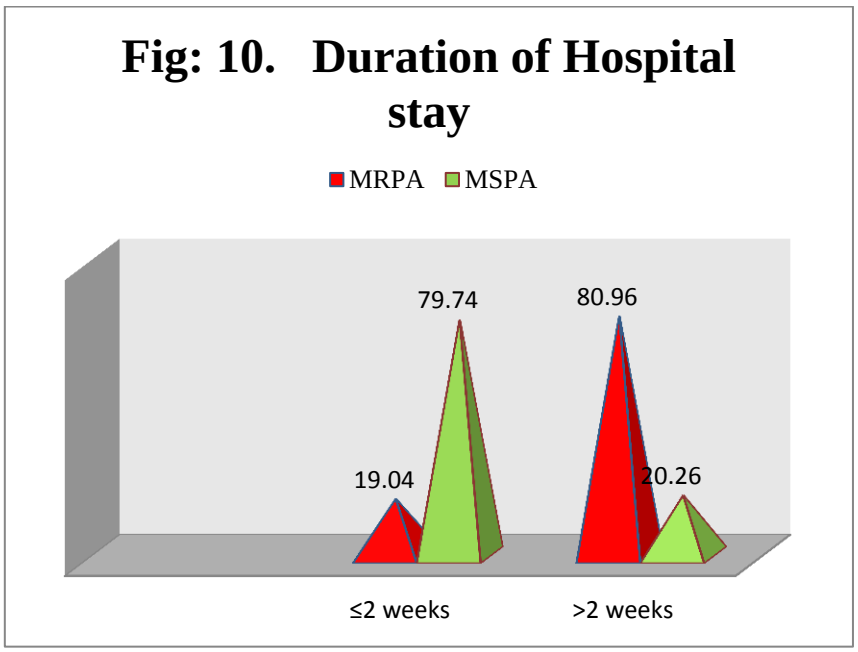


Fig: 10. Duration of Hospital stay

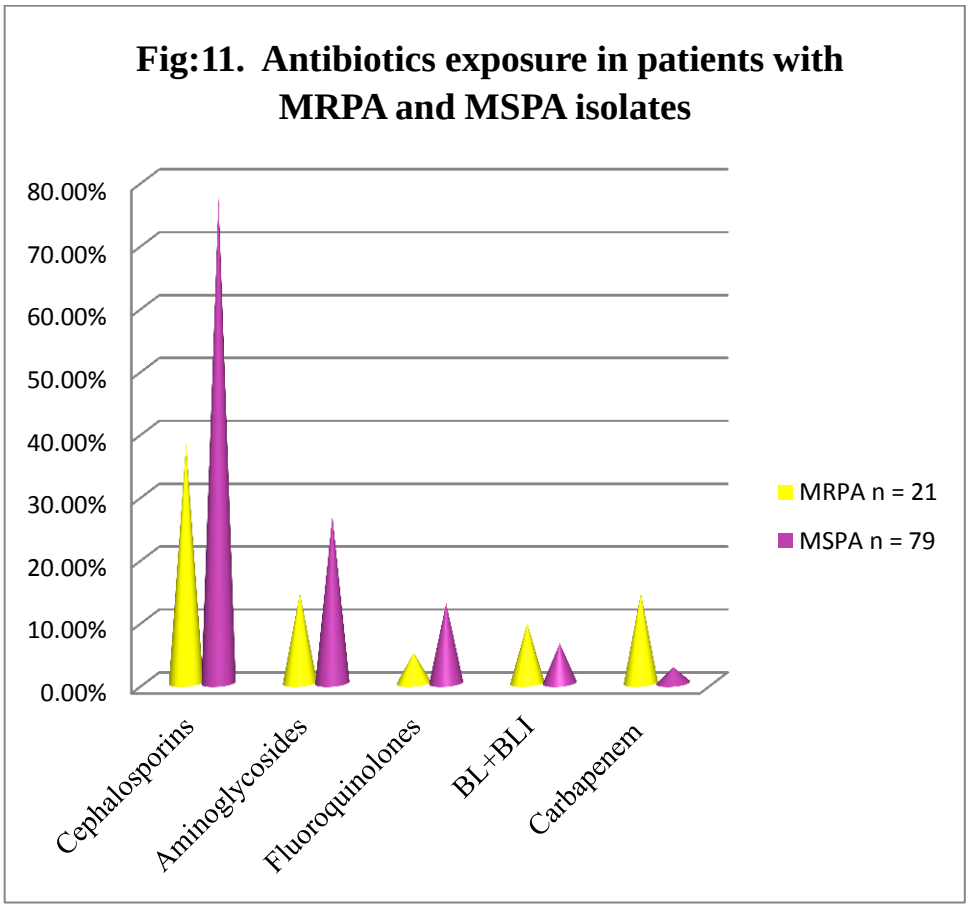
5.15.2. Exposure to antibiotics:

Table: 19 Antibiotic Exposure in patients with MRPA and MSPA

Antibiotics	**MRPA** **n = 21**	**MSPA** **n = 79**	**Total**	**Significance** **p<0.05**
Cephalosporins	8 (38.1%)	61 (77.2%)	69	Significant
Aminoglycosides	3 (14.3%)	21 (26.6%)	24	Non -Significant
Fluoroquinolones	1 (4.8%)	10 (12.7%)	11	Non -Significant
BL+BLI	2(9.5%)	5 (6.3%)	7	Non -Significant
Carbapenem	3 (14.3%)	2 (2.5%)	5	Non -Significant

There was statistically significant difference in exposure to cephalosporins among MRPA and MSPA isolates (P<0.05). There is no significance in the administration of other mentioned antibiotics with the corresponding MSPA and MRPA isolates.(Fig: 11)

Fig:11. Antibiotics exposure in patients with MRPA and MSPA isolates



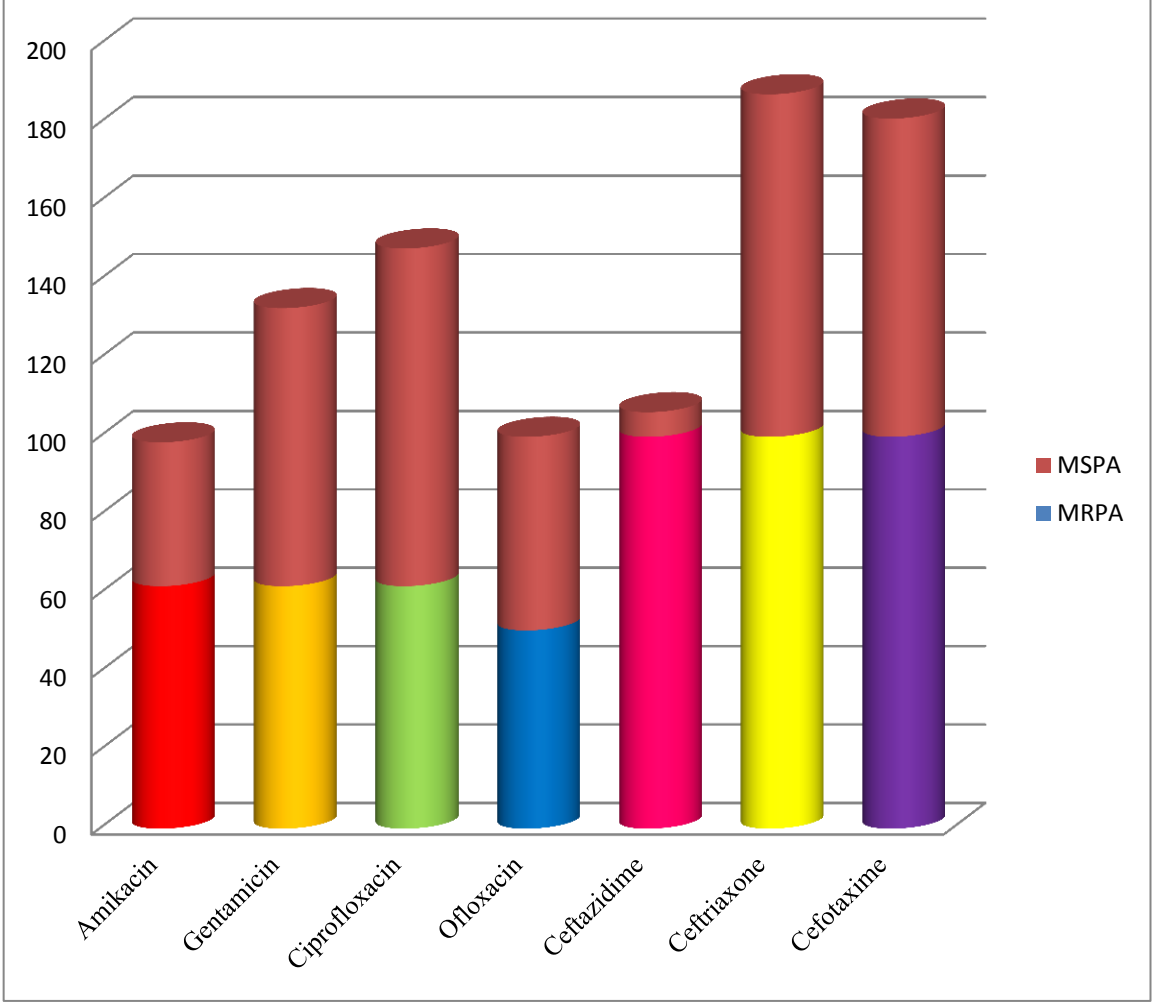
5.16. Resistance pattern to antibiotics in MRPA and MSPA isolates:

Table: 20. Resistance pattern to antibiotics in MRPA and MSPA isolates

Antibiotics	**MRPA n = 21**	**MSPA n = 79**	**Total**	**Significance p<0.05**
Amikacin	13 (61.9%)	29 (36.7%)	42	Significant
Gentamicin	13 (61.9%)	56 (70.9%)	69	Non -Significant
Ciprofloxacin	13 (61.9%)	58 (86.1%)	52	Significant
Ofloxacin	40 (50.6%)	39 (49.4%)	45	Non -Significant
Ceftazidime	21 (100%)	5 (6.3%)	26	Significant
Ceftriaxone	21 (100%)	69 (87.3%)	90	Non -Significant
Cefotaxime	21 (100%)	72 (81.1%)	79	Non -Significant

All the MRPA isolates were found to be resistant to cephalosporins. 61.9% of the MRPA isolates were resistant to Amikacin,Gentamicin and Ciprofloxacin. The resistance to Amikacin,Ciprofloxacin and Ceftazidime were statistically significant. P<0.05. (Table:20, Fig: 12)

Fig:12. Resistance pattern to antibiotics in MRPA and MSPA isolates



6. DISCUSSION

Pseudomonas aeruginosa is the leading cause for the hospital acquired infections. *Pseudomonas aeruginosa* exhibits intrinsic resistance to various antimicrobials. It also shows acquired resistance to anti pseudomonal beta lactams such as piperacillin, cephalosporins and carbapenems. The rise in the resistance to the last resort drug carbapenem has become a major challenge in treating the *P.aeruginosa* infections. Several mechanisms are responsible for the acquired resistance to the β lactam antibiotics in *P.aeruginosa* which includes the production of β lactamases, up regulation of the efflux pump systems and decreased outer membrane permeability. With respect to β lactamases, the metallo beta lactamases are the emerging resistance mechanism in *P.aeruginosa*.

The present study was aimed to identify the prevalence of MBL producing *Pseudomonas aeruginosa* isolated from various specimens in the Department of Microbiology, Tirunelveli medical college.

6.1. Age – Sex wise distribution of the Study group:

The present study showed that the *P.aeruginosa* was isolated more in the age group of 41-70 years (58%). A similar observation was noted by Bashir et al from Kashmir during 2007-2008[8] and Viren A Javiya et al showed 60.7% of *Pseudomonas aeruginosa* were isolated in age group of 41-70 years at a tertiary care hospital, Gujarat during 2006[73].

In the present study *P.aeruginosa* was isolated more commonly in males (73%) when compared to females (27%) with a male: female ratio of 2.7:1. A similar observation was made by Prashanth durwas et al in Bijapur, during 2008-2010 that *Pseudomonas aeruginosa* isolated was more in males with the male to female ratio as 1.8: 1[61]. Unlike the present study, Bashir et al in his study showed that the male: female ratio was 1.2: 1 where the male: female ratio was relatively equal[8].

6.2. Distribution of *Pseudomonas aeruginosa* in wards:

The rate of isolation of *P.aeruginosa* in this study was highest in surgery wards (54%) followed by medicine (18%) and orthopaedics wards (9%). A similar observation was made in a study by Angadi KM et al [74] and Prashant Durwas et al[61].

6.3. Distribution of *Pseudomonas aeruginosa* in clinical isolates:

Most of the specimens collected in this study were pus from wound swab (72%) followed by sputum (19%), vaginal swab (4%) and urine (2%). A study by Mangaiarkkarasi et al [75] and Mahnaz saranghi et al[76] showed that *Pseudomonas aeruginosa* were mostly isolated from the pus samples (59-64%). Shammim mumtaz *et al* in their study obtained 7.3% of the *Pseudomonas aeruginosa* isolates from the vaginal swab[77]. While the present study showed the isolation of *Pseudomonas aeruginosa* is more in pus there are other studies that showed the isolation was highest in specimens like broncho alveolar lavage (Behera et al[78]) and urine (V.javiya

et al[73]). This study indicates that the incidence of pyogenic infection is expected to be highest and was referred from surgical disciplines.

6.4. Resistance pattern of antibiotics in *Pseudomonas aeruginosa* isolates:

About 90% of resistance was seen with cephalosporins like ceftriaxone and cefotaxime in this study. In a study by Nagaveni et al there found 100% resistance to cephalosporins[79]. Many authors like Vikas kumar et al[64], Yousef Irfani et al[80] and Mahnaz et al [76] have reported 52-70% of resistance towards cephalosporins in their study which is in contrast to the present study. Resistance to fluoroquinolones against Ciprofloxacin and Ofloxacin were 71 and 52% respectively. The similar patterns of resistance to fluoroquinolones were reported in a study by Behera et al[78] and Bashir et al[8]. The resistance to aminoglycosides in the present study was 42% which is similar to the study by Saghir et al[81]. Shanthi amudhan et al [40] in their study reported that 100% of the isolates were shown resistance to aminoglycosides and fluorquinolones which is not in line with the results of the current study.

6.5. Screening for MBL production using Meropenem:

In the present study, by disc diffusion test 21% of the entire (100) *Pseudomonas aeruginosa* isolates were resistant to Meropenem (MRPA). As the CLSI guidelines states that the Imipenem should not be used in the detection of carbapenemases, the Meropenem disc is used in this

study. Deepa Bashir et al[8] in their study reported that 13.42% of the entire isolates were resistant to Carbapenem. Vikas kumar et al in their study stated that 19.4% of the *Pseudomonas aeruginosa* isolates were resistant to carbapenem[64]. The findings of the above study are consistent with the present study. Fatemeh fallah et al [30] reported 83% of the *P.aeruginosa* isolates and Horieh Saderi et al [69] reported 69% of *P.aeruginosa* isolates were resistant to Meropenem. The prevalence of resistant isolates was higher in the above studies. Horieh Saderi et al tends to conclude that the reasons for the variability of resistance rates of Meropenem in different studies were not clear though they suggest this difference may reveal the usage of antibiotics in various settings[69]. Similarly Ekta Gupta et al in their study argues that the resistance to the Meropenem was increased in a short period after the frequent use of the antibiotic in their clinical set up [82].

6.6. Distribution of MRPA isolates according to site of infection:

In the present study, 38.1% MRPA isolates were associated with wound infections, 14.5% of the isolates were from vaginitis and diabetic foot, 9.5% were associated with respiratory infections and 4.8% were from urinary tract infections.

The study by Sasikala et al showed that MRPA was predominantly isolated from wound swabs (27%). Twenty per cent of the isolates were obtained from respiratory infections[83]. 43% of the isolates from diabetic foot were shown resistance to Imipenem in a study by Vinodkumar et al[84].

In the present study the isolation of Meropenem resistant *Pseudomonas aeruginosa* was highly associated with surgical interventions like wound debridement, amputation and skin grafting.

6.7. Phenotypic Detection of MBL:

In the present study three different phenotypic methods like CDT, DDST and MBL E test were used to detect the metallo beta lactamase production among the MRPA isolates. (Behera et al.,[78] 2008; Lee et al.,[51] 2003; Arakawa et al., 2000[72]).

6.7.1. Combined disc test(CDT) and Double disc synergy test(DDST):

The present study shows that 14 out of 21 MRPA isolates (66.7%) were detected as MBL producer by the CDT.

Similar results were seen in a study done by Rajput et al that revealed 66.7% of carbapenem resistant strains were MBL producer by CDT[16]. Jamshid Faghri et al in their study showed that CDT detected 42% of Meropenem resistant isolates produced MBL enzyme. This is in contrast with the present study results[85]. The prevalence rate in the above study is low when compared to the present study that may be due to difference in population size or sample size and also the health care practices followed in those areas. In various studies the MBL prevalence in *P.aeruginosa* isolates by CDT were ranging from 14-18% (Hemalatha et al., 2004[86]; Santhosh et al., 2010[87]; Rakesh et al., 2013[88]). Based on a study by Irfan et al, the CDT detected all the 25 carbapenem resistant isolates as MBL producer and

concluded that CDT can be considered as the screening method for the detection of MBL in the Carbapenem resistant isolates[89].

The present study detected 12 out of 21 MRPA isolates (57.1%) as MBL producers by DDST. A study done by Santhosh et al reported that 13 out of 18 resistant isolates were MBL producers by DDST which is similar to our study results.[87] While the present study has the prevalence of about 55% among MRPA isolates, there are many studies which reported higher prevalence of about 70% (Agrawal et al., [62] Fereshteh Shahcheraghi et al.,[37] and S.Aghamiri et al[90]). The dissimilarities in the stated results between the present study and those described above may be due to the difference in geographical regions, difference in kind of infections, the vast usage of antimicrobials, or difference in antibiotic policy in the particular hospitals in this study than those in other studies.

6.7.2. MBL detection by E test:

The MBL E test using MP/MPI (Meropenem and Meropenem with EDTA) in this study detected 10 out of 21 MRPA isolates (47.6%) as MBL producer. Manoharan et al in their study reported 42.6% among the carbapenem resistant isolates were positive for MBL production by E test[91] which is in agreement with the present study results. In contrast to the present study, Bashir et al showed higher prevalence of MBL of about 86% of carbapenem resistant isolates were positive by the E test[8].

6.8. Prevalence of *bla*VIM and *bla*IMP gene in the Meropenem resistant isolates:

The MBL production was detected in 10 out of 21 MRPA isolates (47.6%) for the *bla*VIM gene by PCR in the present study. No *bla*IMP gene was detected in any of the isolates. The genes other than *bla*VIM and *bla*IMP have not found beyond their place of origin and these genes were common all over the world[25]. Mariana Castanheira et al[38], Horieh Saderi et al[39]., M. Shanthi Amudhan et al [40] in their study concluded that the *bla*VIM gene was the most prevalent gene in Asia and India.

Horieh Saderi et al in their study detected 13% of isolates by PCR and only the VIM gene was identified and all of which were positive by phenotypic method. This is in similar with the present study results[39]. A study performed by Khosravi et al in a burn hospital in Iran, reported that all the 8 MBL positive strains contained the VIM gene and the IMP gene was not detected in any of the strains[92]. The greater prevalence of the VIM gene than the IMP gene was revealed in a study by Pitout et al[29] in which 1.6% of isolates was positive for IMP when compared to 43% of isolates that contained VIM gene. In an another study by Mlynarczyk et al showed that the PCR detected 60% of the carbapenem resistant isolates as MBL producer and the gene isolated was *bla*VIM and it accounted for about 70% of the resistant isolates[93]. The prevalence in the above study was higher than the present study and the author concluded that there was an increase in the

prevalence from 7% in 2007 to 15% in 2008 and most of the MBLs belonged to the VIM type. Aghamiri S et al in their study stated that 33% of the entire isolates were producing the MBL and the gene detected was *bla*VIM (33%) and *bla*IMP (9%). The author stated that the isolates negative for MBL gene by PCR may contain genes other than the VIM and IMP responsible for MBL production. The prevalence of VIM gene is more than the IMP gene which is similar to the present study[90]. The similar statements have been quoted in studies by Shanthi amudhan et al[40], Sader HS et al[94] and H. Saderi et al[39].

6.9. Comparison of Phenotypic methods with PCR in the detection of MBL:

Several phenotypic methods such as CDT, DDST and MBL E test are available for the detection of MBL producing *Pseudomonas aeruginosa*. Compared to PCR, the CDT shows the sensitivity and specificity of 90% and 55% respectively.

The result of the present study is in agreement with the study conducted by Manoharan et al that showed the sensitivity and specificity as 87.8% and 53.3%, respectively when compared to the PCR[92]. In a study from Johann D. D. Pitout et al, the CDT showed 100% sensitivity and 96% specificity[29]. The above study shows sensitivity similar to the present study but with the higher specificity.

The DDST in the present study has the sensitivity and specificity of 90% and 72% respectively when compared to PCR for MBL detection.

Bachunde et al, in Maharashtra, 2011 reported the sensitivity of the DDST in their study as 85.7% which is analogous to the present study[95]. S John et al in their study stated that among the tests used for MBL detection DDST is more reliable to CDT and MHT[96]. Yalda khosravi et al in their study reported that the specificity of DDST is higher when compared to CDT[97].

The MBL E test has the sensitivity and specificity of 90% each. Similar results were obtained in the study done by Ting-ting Qu et al with sensitivity and specificity as 85.7% and 100% respectively[72]. Yalda kosravi et al in their study reported that the E test has given the sensitivity and specificity of 100% which is relatively similar to the current study[97]. Kyungon Lee et al in their study reported that the MBL E test is highly sensitive and specific for detecting *bla*IMP and *bla*VIM allele positive isolates of *P.aeruginosa* .[70]

Of the 21 MRPA isolates detected in the present study, the MBL producers by CDT and DDST were 14 and 12 respectively. The kappa value was low for CDT and moderate for DDST which denotes that the phenotypic tests were not effective in MBL detection. The same result has been reported in Franco MR et al[42] Brazil and Fereshteh Shahcheraghi et al [37]. Those strains that were found negative by PCR were considered as false

positive using phenotypic test. The reason for the false positivity may be due to various mechanisms of EDTA such as 1) EDTA increases the membrane permeability that may increase the susceptibility to Meropenem.2) EDTA has bactericidal activity that increases the zone of inhibition to Carbapenems[42,41] that were not associated with the MBL production which would lead to false interpretation of the phenotypic tests.

All the three phenotypic tests used in the present study gives the sensitivity rate of 90% but the specificity rate varies from 55-90%. The sensitivity and specificity for the MBL E test is 90%which is relatively more accurate in detecting the production of MBL when compared to the other phenotypic tests.

In addition the MBL E test has the advantage over the other phenotypic tests, in that the minimum inhibitory concentration can be obtained which will be useful in the treatment of resistant pathogens. Despite the accuracy, the MBL E test is very costly to be used in the clinical laboratory for routine MBL screening procedure. Due to the cost constraints of the MBL E test a simple and cost effective method should be adopted.

The specificity of the DDST is comparatively more than CDT and hence DDST may be considered as a more suitable screening method used for the detection of MBL production in *Pseudomonas aeruginosa*. Those isolates positive by DDST can be further confirmed by the MBL E test if the PCR is not available in the settings of the clinical laboratory.

In the present study not all Meropenem resistant isolates were found to be MBL producers. Similar results have been obtained in various studies such as Alexandre R. Marra et al[41], Ami Varaiya et al[57], Chacko et al[98], Ting ting Qu et al[72] etc. This may be due to other mechanisms of resistance such as mutation in the outer membrane permeability, loss of porins or the upregulation of efflux systems. Jose Manuel Rodriguez Martínez et al[99], Elena Riera et a[100], O. Gutierrez, C et al [101] have done studies to determine these resistance mechanisms other than MBL towards Carbapenem in the *Pseudomonas aeruginosa* isolates.

6.10. Analysis of risk factors:

6.10.1. Duration of hospital stay:

In the present study there is an association between the length of the hospital stay and isolation of MRPA strains. Most of the MRPA isolates were recovered from the patients who had a hospital stay of more than 2 weeks. The mean hospital stay in the present study was 12.46 days.

In a study by Aloush et al, the average length of hospital stay was 17 days among the patients with MRPA and most of the isolates were from wound infections which are concordant with the present study results[102]. Lautenbach et al in a study from two centres stated that the isolation of carbapenem resistant *Pseudomonas aeruginosa* was associated with longer hospital stay than with the susceptible strains[103]. This is in favour with the present study. Eva Morales et al in their study pointed out that the length of

stay (39.0 days) was longer among the patients with resistant *Pseudomonas aeruginosa* which was in accordance with the present study[104].

The length of the hospital stay is considered as an important marker for the morbidity resulting from the infection. Hence the Meropenem resistant *Pseudomonas aeruginosa* can cause increased morbidity.

6.10.2. Exposure to antibiotics:

Exposure to different classes of antibiotics in the patients harbouring MRPA and MSPA strains were analysed. Among these, antibiotics like third generation cephalosporins, aminoglycosides, fluoroquinolones, β lactamase + β lactamase inhibitor combinations and carbapenems, exposure to third generation cephalosporins was statistically significant among corresponding MRPA patients.

Alexandre Prehn Zavascki et al stated in their study that the exposure to β-Lactam was an important risk factor for MBL production in *Pseudomonas aeruginosa* isolates[105].

6.11. Resistance pattern to antibiotics in MRPA:

All the MRPA isolates were resistant to third generation cephalosporins. In studies by Shanthi amudhan et al[40] and Anuradha et al[106] reported that the MRPA isolates were resistant to all antibiotics tested. The third generation cephalosporins are widely used in the community in the treatment of suspected Gram negative infections. This might have affected the susceptibility pattern towards the organism.

About 62% of the MRPA isolates were resistant to amikacin, gentamicin, ciprofloxacin and 50.6% were resistant to ofloxacin. Fereshteh Shahcheraghi et al also reported that most of the MBL producers were resistant to aminoglycosides and fluoroquinolones[37].Since aminoglycosides were the antimicrobial agents most used in association with other antibiotics might be the cause for the resistance.

6.12. Multi drug resistant *Pseudomonas aeruginosa*:

In the present study about 13 out of 21 MRPA isolates were multidrug resistant (MDRPA) i.e. resistant to any of the three classes of antibiotics [45, 46]. Among the 13 MDRPA 8 were MBL producers. All the MDRPA were sensitive to the colistin and 53% of the MDRPA were susceptible to piperacillin-tazobactam and Cefaperazone sulbactam which correlates with the study done by Javiya et al[73].

The rise in the prevalence of MBLs is creating a therapeutic challenge for clinicians and microbiologists. Screening for MBL production as a routine procedure in clinical laboratories with a simple screening test like DDST adds a valuable data to the clinician in appropriate selection of antibiotics and forms a crucial step in large scale monitoring of these emerging resistant strains.

The emergence of resistance to the antibiotics should be constantly monitored so that the antibiotic cycling policies and the occurrence of the newer resistance pattern in the hospital can be determined. There is also a

necessity to highlight on the rational use of antibiotics and strict adherence to the concept of reserve drugs to reduce the inappropriate use of the available antibiotics.

Various strategies like hand washing, environmental decontamination, education to the health care professionals and continuous surveillance will be helpful in attaining a goal of low level of infection with the multidrug resistant pathogens. The hospital infection control guidelines should be formulated strictly to avoid the future spread of these multi-drug resistant strains.

7. SUMMARY

- In the present study the isolation of *P.aeruginosa* was more in the age group of 41-70 years.
- The present study showed *P.aeruginosa* was isolated more in males (73%) than females (27%) with a male : female ratio of 2.7:1.
- The rate of isolation of *P.aeruginosa* was highest in Surgery wards (54%).
- The pus from wound swab constituted the majority of specimens accounting for 72% followed by sputum 19%, vaginal swab 4%, and urine 2%.
- 21% of the entire *P.aeruginosa* isolates were resistant to Meropenem (MRPA).
- Majority of the MRPA infections were associated with wound infections (38.1%).
- Most of the MRPA isolates were recovered from the patients who had a hospital stay of more than 2 weeks which is statistically significant. The mean hospital stay in the present study was 12.46 days.
- Exposure to Cephalosporins was statistically significant among corresponding MRPA patients.

- Among the *Pseudomonas aeruginosa* isolates 10% were MBL producers by PCR.
- The combined disc test(CDT) using Meropenem with EDTA as chelator detected 14(66.7%) isolates of MRPA as MBL producer. The test shows the Sensitivity 90%, Specificity 55%, PPV 64.2% and NPV85.7%.
- The double disc synergy test(DDST) detected about 57.1% of the resistant isolates as MBL producers and the sensitivity, specificity, positive predictive value and negative predictive value of DDST for detecting MBL were 90%, 72%, 90% and 61% respectively.
- The MBL E test in this study detected 47.6% of the resistant isolates as MBL producer and the sensitivity, specificity, positive predictive value and negative predictive value were 90% each.
- The DDST is the simple, cost effective method in the detection of the MBL.
- The E test is relatively accurate in the detection of MBL as close to the PCR.
- All the MRPA isolates were resistant to third generation cephlosporins and about 62% of the MRPA isolates were resistant to Amikacin, Gentamicin, and Ciprofloxacin.

- In the present study 13 isolates were multidrug resistant(MDRPA) i.e resistant to any of the three classes of antibiotics and these isolates were sensitive to colistin which may be the drug of choice for the MDRPA isolates.

8. CONCLUSION

- This study highlights the prevalence of MBL among various clinical isolates and also detects the presence of *bla*VIM and *bla*IMP gene among MRPA.
- The MBL E test is very accurate in detecting the MBL as close to the PCR but with the cost constraints Double disc synergy test is very simple, easy and cost effective that can be easily adopted in the clinical laboratory.
- Early detection of the MBL is crucial to include timely execution of strict infection control practices and treatment with alternative antimicrobials.
- Good antibiotic policy, judicial use of antibiotics, hand hygiene and continuous surveillance will fight against the serious therapeutic challenges faced by the MBL *Pseudomonas aeruginosa*.
- The colistin has become the last resort drug that can be used for the multidrug resistant *Pseudomonas aeruginosa*.

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ANNEXURE – 1

1. Nutrient agar medium:

Composition

Ingredients gram/liter

Peptic digest of Animal Tissue 5.00

Sodium Chloride 5.00

Beef Extract 1.50

Yeast Extract 1.50

Agar 15.00

Twenty-eight grams of dehydrated nutrient agar medium was added to 1000 ml of cold distilled water in a flask and boiled to dissolve the medium completely. The medium was then sterilized in an autoclave at 121^0C and 15 lbs pressure for 15 minutes. The sterile media were stored in a refrigerator at 4^0C for future use.

2. MacConkey agar medium:

Composition - Ingredients gram/liter

Peptone 19.0

Lactose 10.0

NaCl 5.0

Na- Deoxycholate 1.0

Neutral Red 0.03

Crystal Violet 0.001

Agar 15.0

Fifty-two grams of dehydrated MacConkey agar medium was suspended in 1000 ml of cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes.

3. Blood agar medium

Composition

Ingredients gram/liter

Heart infusion 500.00

Tryptose 10.00

Sodium chloride 5.00

Agar 15.00

Forty grams of the dehydrated blood agar medium was suspended in 1000 ml cold distilled water in a flask and boiled to dissolve the medium completely. It was then sterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes. The autoclaved materials were allowed to cool to a temperature of 45^0C in a water bath. Defibrinated 5-10% sheep blood was then added to the medium aseptically and distributed to sterile petri dishes. Sterile media was stored in refrigerator at 4^0C for future use.

4. Muller Hinton agar medium

Composition

Ingredients gram/liter

Beef dehytrated infusion 300

Casein hydrolysate 17.50

Starch agar 17.00

Agar 17.00

Thirty-eight grams of dehydrated Mueller Hinton agar medium was suspended in 1000 ml cold distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121^0C and 15 lbs pressure for 15 minutes. The autoclaved media was stored in the refrigerator and used later.

5. McFarland Standard (0.5):

Reagents:

Sulphuric acid, 1%: To 100 ml of distilled water,1 ml of conc.sulphuric acid is added. Barium chloride, 1.175%: To 100 ml of distilled water, 1.175gm of barium chloride is added and mixed well.

To prepare McFarland 0.5 standards:

To 85 ml of 1% conc.sulphuric acid, 0.5 ml of Barium chloride is added in a flask while constantly swirling the flask. Bring to 100 ml with 1% conc.sulphuric acid. Aliquot in test tubes and cap tubes tightly. Store in the dark at room temperature for 3 months or longer.

PROFORMA

Name :

Age :

Sex :

OP/IP No :

Lab No :

Ward :

Complaints :

Clinical diagnosis :

Nature of Specimen :

Duration of hospital stay :

Antibiotics administered :

Investigation :

Biochemical tests : Indole, Citrate, Urease, Triple sugar iron, Catalase, Oxidase

Antibiogram :

Piperacillin, Amikacin, Ceftriaxone, Cefotaxime, Ceftazidime, Ciprofloxacin, Ofloxacin, Gentamicin, Meropenam, Piperacillin with Tazobactam, Cefoperazone and sulbactam, Colistin.

Screening test with meropenam

Combined disc test

Double disc synergy test

E test

RT-PCR

MASTER CHART

S.NO	MICRO NO	Age	SEX	Ward	DIAGNOSIS	HOSP. STAY DURATION	SPECIMEN	Ceftazidime(30µg)	Cefotaxime(30µg)	Ceftriaxone(30µg)	Gentamicin(10µg)	Amikacin(30µg)	Piperacillin	ciprofloxacin	ofloxocin	Meropenem(10µg)	colistin	Cefperazone-Sulbactam	Piperacillin-Tazobactam	(CDT) MERO+EDTA	(DDST) MERO+EDTA	E TEST	PCR (IMP/VIM GENE)	Cephalosporins	BL+BLI	Aminoglycoside	Fluoroquuinolone	carbapenem	OUTCOME
1	5129	63	F	FS	ABSCESS	18	PUS	R	R	R	R	R	R	R	R	R	S	S	S	P	P	N	N	Y	N	N	Y	N	IMPROVED
2	5185	45	M	MS	non healing ulcer	14	PUS	S	R	S	R	S	S	S	S	S	NA							Y	N	Y	N	N	IMPROVED
3	5198	41	M	MS	BURNS	10	PUS	S	S	S	S	R	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
4	5254	45	M	MS	WOUND SEPSIS	14	PUS	S	R	R	R	R	S	R	R	S	NA							N	N	N	Y	N	IMPROVED
5	5269	60	M	S	Diabetic foot	16	PUS	S	R	S	R	S	S	S	S	S	NA							Y	N	Y	N	N	IMPROVED
6	5349	50	F	S	DM. Cellulitis	8	PUS	S	R	R	R	R	S	R	R	S	NA							Y	N	N	N	N	IMPROVED
7	5352	37	M	M	PNEUMONIA	7	SPUTUM	S	R	R	S	R	S	R	R	S	NA							N	Y	Y	N	N	IMPROVED
8	5354	67	F	M	PNEUMONIA	6	SPUTUM	S	R	R	R	S	S	S	S	S	NA							Y	N	N	N	N	IMPROVED
9	5496	60	M	M	WOUND INF	8	PUS	S	S	S	S	S	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
10	5818	57	M	TB	TB	7	SPUTUM	S	R	R	R	R	S	S	R	S	NA							N	N	N	Y	N	IMPROVED
11	5819	51	F	TB	TB	8	SPUTUM	S	R	R	S	R	S	S	S	S	NA							Y	N	Y	N	N	IMPROVED
12	5830	27	F	LW	PNEUMONIA	7	SPUTUM	S	R	R	R	S	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
13	5836	55	M	S	DM	10	PUS	S	R	R	R	R	S	R	R	S	NA							Y	N	N	N	N	IMPROVED
14	5838	77	M	S	WOUND SEPSIS	14	PUS	S	S	R	S	S	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
15	5853	59	M	S	Necrotising fascitis	16	PUS	R	R	R	S	S	R	R	S	R	S	S	S	P	P	P	P	Y	N	N	N	N	IMPROVED
16	5844	45	M	O	WOUND SEPSIS	17	PUS	R	R	R	R	S	R	R	S	R	S	S	S	N	N	N	N	N	Y	N	N	N	IMPROVED
17	5986	50	M	M	PNEUMONIA	7	SPUTUM	R	R	R	S	S	R	RR	S	S	NA							N	N	Y	N	N	IMPROVED
18	5989	28	M	O	WOUND SEPSIS	12	PUS	S	R	R	R	S	S	S	S	S	NA							Y	N	N	N	N	IMPROVED
19	2933	2	M	PICU	SEPSIS	24	PUS	R	R	R	R	S	R	R	S	R	S	S	S	N	N	N	N	N	N	Y	N	N	IMPROVED

20	6159	25	F	LW	PNEUMONIA	5	SPUTUM	S	R	R	R	S	R	R	S	S	NA							Y	N	Y	N	N	IMPROVED
21	6184	32	F	LW	VAGINITIS	17	VAGINALS WAB	R	R	R	R	R	R	R	R	R	S	R	R	P	P	P	P	Y	N	N	N	N	IMPROVED
22	6217	55	M	M	BRONCHIECTASIS	21	SPUTUM	R	R	R	S	S	R	S	S	R	S	S	S	N	N	P	P	Y	N	N	N	N	IMPROVED
23	6219	65	M	S	DIABETIC FOOT	20	PUS	R	R	R	R	S	R	R	S	R	S	S	S	N	N	N	N	N	N	N	N	Y	IMPROVED
24	6251	65	M	S	DIABETIC GANGRENE	21	PUS	S	R	R	S	S	R	R	S	S	NA							Y	N	N	N	N	IMPROVED
25	6242	11	M	P.S	ABSCESS	7	PUS	R	R	R	R	S	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
26	6302	25	F	LW	PUO	16	VAGINALS WAB	R	R	R	R	R	R	R	R	R	S	R	R	P	P	P	P	Y	N	Y	N	N	IMPROVED
27	6314	40	M	O1	WOUND SEPSIS	17	PUS	R	R	R	S	S	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
28	6348	44	M	M	PNEUMONIA	16	SPUTUM	R	R	R	R	R	R	R	R	R	S	S	S	P	P	N	N	Y	N	N	N	N	IMPROVED
29	6359	49	M	ENT	CSOM	8	AURAL PUS	R	R	R	R	R	R	R	R	R	S	S	S	P	P	P	P	Y	N	Y	N	N	IMPROVED
30	6360	29	M	O	infected fracture wound	19	PUS	R	R	R	R	R	R	R	R	R	S	R	R	P	P	P	P	N	N	N	N	Y	IMPROVED
31	6429	57	M	O	WOUND SEPSIS	17	PUS	R	R	R	R	S	R	R	S	R	S	S	S	N	N	N	N	N	N	Y	N	N	IMPROVED
32	6434	65	F	FS	DM. Cellulitis	14	PUS	S	R	R	R	S	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
33	6438	69	M	S	DM-Diabetic foot	13	PUS	S	R	R	S	S	R	S	R	S	NA							Y	N	Y	N	N	IMPROVED
34	6330	55	M	S	DM	16	PUS	S	R	R	R	S	S	R	R	S	NA							N	N	Y	N	N	IMPROVED
35	6333	58	M	S	BURNS	21	PUS	S	R	R	R	S	R	R	R	S	NA							Y	N	Y	N	N	IMPROVED
36	6614	44	M	M	PNEUMONIA	6	SPUTUM	R	R	R	R	S	R	S	R	S	NA							Y	N	Y	N	N	IMPROVED
37	8152	65	M	U	PROSTITIS	17	URINE	R	R	R	S	S	S	R	R	R	S	S	S	N	N	N	N	N	N	Y	N	N	IMPROVED
38	8360	32	F	U	UTI	6	URINE	S	R	R	S	R	R	S	S	S	NA							Y	N	Y	N	N	IMPROVED
39	13	25	F	LW	VAGINITIS	16	VAGINALS WAB	R	R	R	R	S	S	S	S	R	S	S	S	N	N	N	N	Y	N	N	N	N	IMPROVED
40	25	53	M	M	PLEURAL EFFUSION	15	PLEURAL FLUID	R	R	R	R	S	S	R	S	S	NA							Y	N	Y	N	N	IMPROVED
41	55	2	M	M	HYDROCEPHALUS	14	CSF	S	R	R	S	R	R	R	R	S	NA							N	Y	Y	N	N	IMPROVED
42	56	20	M	S	BURNS	18	PUS	S	R	R	S	R	R	S	S	S	NA							Y	N	Y	N	N	IMPROVED
43	98	69	M	S	DM	21	PUS	R	R	R	R	R	R	R	R	R	S	S	S	P	N	N	N	N	N	Y	N	N	IMPROVED
44	122	60	M	O	WOUND SEPSIS	20	PUS	S	R	R	S	S	S	R	S	S	NA							Y	N	Y	N	N	IMPROVED
45	124	28	F	O	WOUND SEPSIS	17	PUS	R	R	R	R	R	R	R	R	R	S	S	S	P	P	P	P	N	N	N	N	Y	IMPROVED
46	132	70	F	S	DM	15	PUS	R	R	R	R	R	R	R	R	R	S	R	R	P	P	P	P	N	N	Y	N	N	IMPROVED
47	134	12	M	ENT	CSOM	6	PUS	S	R	R	R	S	S	R	S	S	NA							Y	N	N	N	N	IMPROVED

48	190	75	M	S	PNUMONITIS	5	SPUTUM	S	R	R	S	R	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
49	203	68	M	S	PYOCELE	21	PUS	S	R	R	R	S	R	R	S	S	NA							N	N	Y	N	N	IMPROVED
50	217	60	F	S	ABSCESS	7	PUS	S	R	R	S	R	S	S	R	S	NA							Y	Y	N	N	N	IMPROVED
51	277	55	M	M	PNEUMONIA	8	SPUTUM	S	R	R	R	S	R	R	S	S	NA							Y	N	N	N	N	IMPROVED
52	298	30	M	S	WOUND SEPSIS	15	PUS	R	R	R	R	R	R	R	R	R	S	S	S	P	P	P	P	N	N	Y	N	N	IMPROVED
53	333	73	M	LEP	WOUND SEPSIS	14	PUS	S	R	R	R	S	S	R	S	S	NA							Y	N	Y	N	N	IMPROVED
54	337	65	F	S	DM	12	PUS	S	R	R	S	R	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
55	345	12	F	ChS	SSI	18	PUS	S	R	R	R	S	R	S	R	S	NA							Y	N	Y	N	N	IMPROVED
56	368	68	M	S	colostomy drain infetion	15	PUS	S	R	R	R	S	R	R	S	S	NA							N	Y	N	N	N	IMPROVED
57	395	40	F	S	WOUND SEPSIS	14	PUS	S	R	R	R	R	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
58	403	70	M	S	WOUND SEPSIS	17	PUS	S	R	R	R	S	S	S	R	S	NA							Y	N	N	Y	N	IMPROVED
59	404	45	M	S	WOUND SEPSIS	18	PUS	S	R	R	R	R	R	R	R	S	NA							Y	N	N	N	N	IMPROVED
60	408	35	F	S	post op wound infection	10	PUS	S	R	R	R	S	R	R	R	S	NA							Y	N	N	N	N	IMPROVED
61	420	61	M	M	PNUMONIA	8	SPUTUM	S	R	R	S	R	S	R	S	S	NA							N	N	Y	N	N	IMPROVED
62	429	55	M	S	WOUND SEPSIS	10	PUS	S	R	R	R	S	R	R	S	S	NA							Y	N	N	N	N	IMPROVED
63	483	9	F	ChS	WOUND SEPSIS	10	PUS	S	R	R	R	R	S	S	S	S	NA							Y	N	Y	N	N	IMPROVED
64	497	80	M	S	WOUND SEPSIS	13	PUS	S	R	R	R	S	R	R	S	S	NA							Y	N	N	Y	N	IMPROVED
65	526	45	F	S	WOUND SEPSIS	12	PUS	S	R	R	R	R	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
66	537	27	M	O	INFECTED FRACTURE	17	PUS	S	S	R	R	S	S	S	R	S	NA							Y	N	Y	Y	N	IMPROVED
67	545	60	M	S	WOUND SEPSIS	11	PUS	S	S	R	S	S	S	R	S	S	NA							N	N	N	Y	Y	IMPROVED
68	554	60	M	M	PNEUMONIA	9	SPUTUM	S	R	R	R	R	R	R	R	S	NA							Y	N	Y	N	N	IMPROVED
69	558	42	M	M	PNEUMONIA	8	SPUTUM	S	S	R	R	S	S	R	R	S	NA							Y	N	N	N	N	IMPROVED
70	570	65	M	S	WOUND SEPSIS	12	PUS	S	S	R	R	S	R	S	S	S	NA							Y	N	N	Y	N	IMPROVED
71	640	70	M	S	PYOCELE	17	PUS	R	R	R	R	R	R	R	R	R	R	R	R	P	P	N	N	N	N	Y	N	N	IMPROVED
72	668	65	M	S	WOUND SEPSIS	20	PUS	R	R	R	R	R	R	R	R	R	R	S	S	P	N	N	N	N	Y	N	N	N	IMPROVED
73	694	37	F	ENT	CSOM	10	PUS	R	R	R	R	R	R	R	R	R	R	R	R	P	P	P	P	N	N	Y	N	N	IMPROVED
74	736	39	M	S	WOUND SEPSIS	8	PUS	S	R	R	S	S	S	S	S	S	NA							Y	N	N	N	N	IMPROVED
75	743	34	F	LW	PNUMONIA	5	SPUTUM	S	R	R	R	R	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
76	747	19	F	BURNS	BURNS	5	PUS	S	R	R	R	S	S	S	S	S	NA							N	Y	Y	N	N	IMPROVED
77	761	70	M	S	WOUND SEPSIS	15	PUS	S	R	R	R	S	S	S	S	S	NA							Y	N	N	N	N	IMPROVED
78	818	60	M	S	WOUND SEPSIS	16	PUS	S	R	R	R	R	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED

79	875	59	M	S	WOUND SEPSIS	21	PUS	S	R	R	R	S	S	R	R	S	NA							Y	N	N	N	N	IMPROVED
80	904	39	M	CAS	ABSCESS	6	PUS	S	R	R	R	S	S	R	R	S	NA							N	N	N	Y	Y	IMPROVED
81	909	75	F	S	DIABETIC FOOT	8	PUS	S	R	R	R	S	R	S	S	S	NA							Y	N	Y	N	N	IMPROVED
82	915	28	M	CAS	ABSCESS	5	PUS	S	R	R	R	R	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
83	917	35	M	S	WOUND SEPSIS	7	PUS	S	R	R	S	S	R	R	R	S	NA							Y	N	N	N	N	IMPROVED
84	951	39	M	CAS	ABSCESS	5	PUS	S	R	R	R	S	S	S	R	S	NA							Y	N	Y	N	N	IMPROVED
85	977	55	M	S	WOUND SEPSIS	13	PUS	S	R	R	R	S	R	R	S	S	NA							N	N	N	N	N	IMPROVED
86	996	55	M	S	WOUND SEPSIS	17	PUS	S	R	R	R	R	S	R	S	S	NA							Y	N	N	N	N	IMPROVED
87	1064	67	M	M	PNEUMONIA	7	SPUTUM	S	R	R	R	R	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
88	1078	40	M	ORTHO	WOUND SEPSIS	12	PUS	S	R	R	R	S	S	S	S	S	NA							N	N	Y	N	N	IMPROVED
89	1138	54	M	M	COPD	15	SPUTUM	S	R	R	R	S	R	R	R	S	NA							Y	N	N	N	N	IMPROVED
90	1184	35	F	S	PNEUMONIA	11	SPUTUM	S	R	R	R	R	S	R	R	S	NA							Y	N	Y	N	N	IMPROVED
91	1203	86	M	M	DM	18	PUS	S	R	R	R	S	S	S	R	S	NA							Y	N	N	Y	N	IMPROVED
92	1145	29	M	S	BURNS	4	PUS	S	R	R	S	S	R	S	S	S	NA							Y	N	Y	N	N	IMPROVED
93	1153	35	F	S	DM	13	PUS	S	R	R	R	R	S	R	R	S	NA							N	N	Y	N	N	IMPROVED
94	1167	35	M	S	ABSCESS	6	PUS	S	R	R	S	S	R	R	R	S	NA							Y	N	N	N	N	IMPROVED
95	1205	65	M	S	DM	7	PUS	S	R	R	S	R	S	R	R	S	NA							N	N	N	N	N	IMPROVED
96	1228	62	F	LW	VAGINITIS	5	VAGINALS WAB	S	R	R	S	S	S	S	S	S	NA							Y	N	N	N	N	IMPROVED
97	1287	59	M	BURNS	BURNS	8	PUS	S	R	R	S	R	S	R	S	S	NA							Y	N	Y	N	N	IMPROVED
98	1300	27	M	S	WOUND SEPSIS	9	PUS	S	R	R	S	S	S	R	R	S	NA							N	N	N	Y	N	IMPROVED
99	1319	65	M	S	WOUND SEPSIS	12	PUS	S	R	R	S	R	S	S	S	S	NA							Y	N	N	N	N	IMPROVED
100	1367	47	M	S	WOUND SEPSIS	15	PUS	S	R	R	S	S	R	R	R	S	NA							Y	N	N	N	N	IMPROVED